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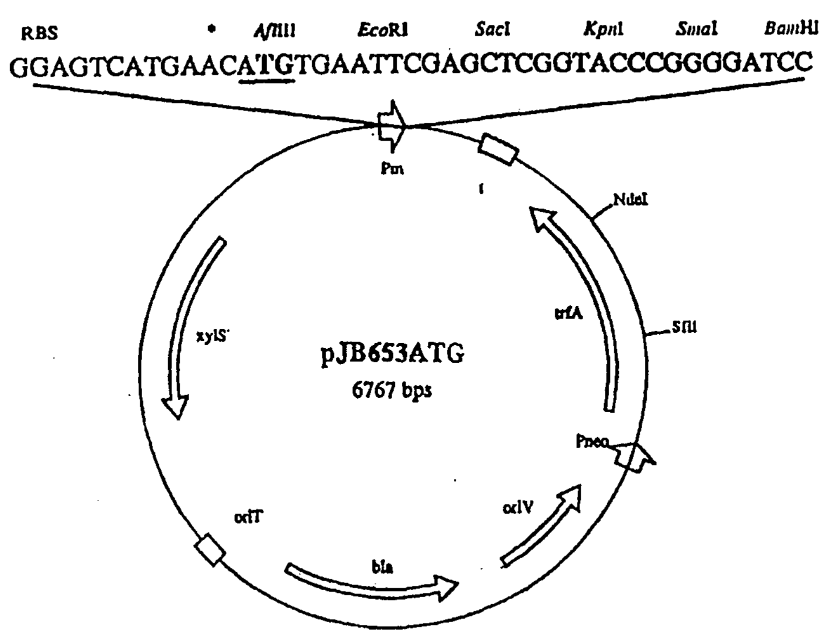

(57) Abstract

The present invention provides an expression vector comprising an RK2 minimum replicon together with an expression cassette comprising the regulatory functions of a TOL plasmid, and, in particular, an expression vector comprising a RK2 minimum replicon together with a promoter *Pm* and/or *Pu* and a corresponding regulatory gene *xylS* and/or *xylR* as derived from a TOL plasmid. Such expression vectors may be used to express desired genes in a wide range of gram negative and gram positive bacterial hosts.

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NOVEL EXPRESSION VECTORS

The present invention relates to novel expression vectors for expressing desired genes within a range of bacterial hosts and, in particular, to expression vectors based on the RK2 replicon and the TOL plasmid regulatory functions.

The cloning and expression of genes is a central tool in biotechnology. Traditionally, genes have been cloned and expressed in enteric bacteria, most notably *E.coli*, which for a long time was regarded as the most useful host for gene cloning. However, the inability of *E.coli* to express some biological properties, for example certain metabolic activities, or to carry out appropriate modifications and processing of certain gene products, has encouraged the development of alternative host-vector systems, in particular for different hosts. The use of non-enteric bacteria for basic and applied molecular research has extended the need for well characterised vector systems for such organisms. Thus, vector systems have been designed which are specific for the bacterial species of interest, e.g. soil bacteria. However, a more useful approach would be to design vectors which may be used across a broad range of microbial hosts, and work in recent years has been directed to this.

In addition, expression of foreign genes, and indeed over expression of native genes, can significantly perturb the physiology of the host cell and constitute a strong selective pressure for elimination or inactivation of the cloned genes. Vectors in which the expression of cloned genes can be regulated and controlled have therefore become increasingly important.

The present invention is directed towards meeting this continuing need for new and improved expression

vectors for the controlled expression of genes in a wide range of hosts. In particular, it has been found that efficient and controlled expression of cloned genes in a broad range of hosts may be achieved by constructing expression vectors which combine the replicon from the RK2 plasmid family with the expression regulatory functions of the TOL plasmids.

In its broadest aspect, the present invention thus provides an expression vector comprising an RK2 minimum replicon together with an expression cassette comprising the regulatory functions of a TOL plasmid.

As used herein the term "expression cassette" refers to a nucleotide sequence encoding or comprising the various functions required to express a DNA sequence, notably the promoter-operator functions and the associated regulatory sequences required for expression from that promoter, e.g translational and transcriptional control elements and/or sequences encoding regulatory proteins, which may act to regulate expression, for example at the level of the promoter.

RK2 is a well-characterised naturally occurring 60Kb self-transmissible plasmid of the IncP incompatibility group well known for its ability to replicate in a wide range of gram-negative bacteria (Thomas and Helinski, 1989, in Promiscous Plasmids in Gram-negative bacteria (Thomas, C.M., Ed.) Chapter 1, pp 1-25, Academic Press Inc (London) Ltd, London). It has been determined that the minimal replicating unit of RK2 consists of two genetic elements, the origin of vegetative replication (*oriV*), and a gene (*trfA*) encoding an essential initiator protein (TrfA) that binds to short repeated sequences (iterons) in *oriV* (Schmidhauser and Helinski, 1985, J. Bacteriol. 164, 446-455; Perri et al., 1991, J. Biol. Chem; 266, 12536-12543). This minimal replicating unit is termed the so-called "RK2 minimum replicon", and has been extensively characterised and studied in the literature. A wide range of replicons (termed "mini-RK2 replicons") and

cloning vectors based on the RK2 minimum replicon or on derivatives of the RK2 plasmid have been prepared and described in the literature (see, for example, Li et al., 1995, J. Bacteriol. 177, 6866-6873; Morris et al., J. Bacteriol., 177, 6825-6831; Franklin and Spooner, in Promiscous Plasmids in Gram-negative bacteria (Thomas, C.M., ed) Ch. 10, pp 247-267, Academic Press Inc. (London) Ltd., London; Haugan et al., 1992, J. Bacteriol 174:7026-7032; and Valla et al., 1991, Plasmid, 25, 131-136).

The TOL plasmids are another series of well-characterised naturally occurring plasmids and their derivatives, which occur in *Pseudomonas* sp. and which encode the enzymes required for the catabolism of toluene and xylenes (for a review see Assinder and Williams 1990, Adv. Microb. Physiol., 31, 1-69).

The catabolic genes of TOL plasmids are organised in two operons, an upper pathway operon (OP1) encoding genes and regulatory sequences required for the oxidation of aromatic hydrocarbons to aromatic carboxylic acids, and a lower, or *meta* pathway operon (OP2) necessary for the oxidation and ring clearage of the aromatic nucleus of aromatic carboxylic acids, giving rise to intermediates which are channelled into the intermediary metabolism. The expression of the two operons is controlled by two positive regulatory proteins XylR and XylS, in the presence of the corresponding substrate ligands toluene/xylene and benzoate/toluate respectively. Activated XylR stimulates transcription from the Promoter *Pu* of the upper pathway operon, whereas activated XylS induces the *meta* pathway operon from the promoter *Pm*. XylR may also induce the promoter *Ps* of the *xylS* gene (see Assinder and Williams, Supra). A regulatory cassette based on the *xylR* gene and *Pu* promoter has been described and used to prepare expression vectors which enable regulated gene expression induced by aromatic hydrocarbons (Keil and Keil, 1992, Plasmid, 27, 191-

199). However, it has not previously been proposed to combine the TOL regulatory functions *Pu/xylR* or *Pm/xylS* with an RK2-based replicon within an expression vector construct.

Viewed from a further aspect, the present invention thus provides an expression vector comprising a RK2 minimum replicon together with a promoter *Pm* and/or *Pu* and a corresponding regulatory gene *xylS* and/or *xylR* as derived from a TOL plasmid.

In such expression vectors of the invention the catabolic genes of the TOL plasmids, encoding the enzymes of the metabolic pathway, are generally absent. Especially, the full complement of catabolic structural genes, in any one, or both, of the operons, are absent.

The novel vectors of the invention allow the regulated expression of cloned genes in a wide range of host cells.

As mentioned above, the RK2 replicon has been well studied and its complete nucleotide sequence is reported (Pansegrau et al., 1994, J. Mol. Biol., 239, 623-633). Thus, sources for the RK2 minimum replicon are well established and readily available. Hence, for example, the RK2 minimum replicon may be derived from the parental plasmid RK2 or from any of the vast number of derivatives or mini RK2 plasmids described and available from the literature (see e.g. Li et al; Morris et al., Franklin and Spooner; Haugen et al; and Valla et al., Supra). As exemplary of a suitable source plasmid for the minimum RK2 replicon may be mentioned plasmid pFF1 (Durland et al., 1990, J. Bacteriol, 172, 3859-3867), but many other source plasmids are available and could be used. The separate elements of the minimum replicon, *oriV* and the *trfA* gene may be isolated from the same source together or separately or from separate sources.

Likewise, any of the TOL plasmids and their derivatives widely known and described in the literature could be used as the source of the TOL regulatory functions (see e.g. Assinder and Williams, Keil and

Keil, Supra and Mermod et al., 1986, J. Bacteriol., 167, 447-454). Indeed, a number of plasmids are known in the literature which have TOL genes inserted, and any of these could be used as the source of the TOL regulatory functions for the present invention. The regulatory genes *xylR* and/or *xylS* may be inserted together with the *Pu* and/or *Pm* promoter from the same source or the promoter and regulatory gene may be derived independently from separate sources. Thus, for example a *Pm* promoter may be derived from plasmid pERD21, (a RSF1010-based replicon, Ramos et al., 1988, Febs Letters, 226, 241-246), a *Pu* promoter may be derived from plasmid pRD579 (an R1-based replicon, Dixon et al., 1986, Molec. Gen. Genet. 203, 129-136), a *xylS* gene may be derived from plasmid pERD839 (a plasmid based on the RSF1010 replicon, Michan et al., 1992, 267, 22897-22901; this publication also mentions other plasmids which may be the source of *xylS* genes, e.g. pERD103 for wild-type *xylS*) and a *xylR* gene may be derived from plasmid pTS179 (a pACYC184 replicon, Inouye et al., 1983, J. Bacteriol., 155, 1192-1199. Alternatively the *Pu/xylR* expression cassette of Keil and Keil (supra) could be used. These sources are however only exemplary, and a number of alternative source plasmids could be used, selected from among the vast number known in the literature.

Techniques for excising the desired nucleotide sequences containing the TOL promotor and/or regulatory regions or the RK2 minimum replicon functions from a selected source and introducing them into an expression vector or intermediate construct are well known and standard in the art, and are described for example in Sambrook et al., 1989, Molecular cloning; a laboratory manual, 2nd Edition, Cold Spring Harbour Laboratory Press, Cold Spring Harbour, N.Y.

As will be described in more detail in the Examples below, it is convenient to isolate the desired sequences from a selected source and introduce them, using

techniques standard in the art, into a series of intermediate constructs, which may be plasmids, introducing or adding or deleting elements to arrive at the expression vectors of the invention.

As used herein the terms "RK2 minimum replicon" and "TOL regulatory functions" and indeed the separate genetic elements "*oriV*", "*trfA*" "*Pm*", "*Pu*", "*xylS*" and "*xylR*" include not only the native or wild-type functions as they appear in the original, parental or archetypal source plasmids but also any modifications of the functions, for example by nucleotide addition, deletion, or substitution or indeed chemical modification of the nucleotides, which occur naturally, e.g. by allelic variation or spontaneous mutagenesis, or which are introduced synthetically. Techniques for modification of nucleotide sequences are standard and well known in the literature and include for example mutagenesis, e.g. the use of mutagenic agents or site-directed mutagenesis. PCR may also be used to introduce mutations. Appropriate or desired mutations, may for example be selected by mutant screening of the genetic element in question e.g. the promoter.

Thus, modifications may be introduced into the *trfA* gene, for example, to increase copy number of the vector within a host cell, or to achieve temperature sensitive replication. Such modifications have been described in the literature. The copy number of RK2 within *E.coli* is usually estimated to be 5-7 plasmids per chromosome. However, this may be elevated in both *E.coli* and other bacteria by certain point mutation in the *trfA* gene, which may lead to copy numbers up to 23-fold higher than normal. Such "copy up" or "*cop* mutations" are described for example in Durland et al., 1990, J. Bacteriol, 172, 3859-3867; Haugan et al., 1992 supra; and Haugan et al., 1995, Plasmid, 33, 27-39. *Cop* mutations have been shown to be most effective in increasing copy numbers in *E. coli*; in other bacteria, such high copy numbers may not be tolerated. Nonetheless, *cop* mutations in the *trfA*

gene may be used to increase expression in bacterial species beyond *E.coli*.

Results have shown that expression of genes from the vectors of the invention may be modified by changing the copy number of the vectors. This is a unique and useful feature, which could be used, for example, to reduce expression due to the formation of inclusion bodies. A lower copy number of *cop* plasmids may also be used to reduce background and gene expression, in the absence of inducer. This may be particularly useful if the gene product is toxic to the host cell.

Studies have shown that *cop* mutations in *trfA* tend to be localised between the *Nde* I and *Sfi* I sites in *trfA*, and that *cop* mutations may readily be prepared by exchanging the *Sfi* I/*Nde* I fragment internally in the *trfA* gene, and straight-forward one-step cloning procedures (see Haugan et al., 1995, supra). It is therefore advantageous to keep the *Sfi* I and *Nde* I sites in *trfA* unique in the vectors of the invention.

Mutations may also be introduced into *trfA* to render the replication of RK2-*oriV* plasmids temperature sensitive, as described for example in Valla et al., 1991 and Haugan et al., 1992, supra. The *trfA* gene is known to encode two related proteins of 44 and 33 kDa that are produced by independent translation initiation at two start codons within the same open reading frame (Shingler and Thomas, 1984, J. Mol. Biol. 175, 229-249). Mutations may be introduced using analogous techniques to alter other functional properties of these proteins.

Within the scope of the invention, vectors may be created which permit regulated expression of *trfA*, permitting replication of the vector to be controlled. Thus, for example, vectors have been constructed in which *trfA* is placed under control of the *Pm* promoter. This may be achieved simply by deleting *trfA* from its original position in a vector of the invention such as pJB653ATG (see Example 1) and inserting it downstream of *Pm*. Vectors in which *trfA* is under the control of *Pu*

may analogously be constructed. The useful property of such vectors is that they replicate as long as the promoter is kept induced by the presence of the external inducer (i.e. an aromatic hydrocarbon), while replication is blocked in the absence of the inducer; a certain minimum amount of TrfA protein is required for replication and if insufficient TrfA is expressed the vectors cannot replicate, which generally occurs in the absence of inducer (although this is dependent on cell growth temperature - see Table 8).

Vectors which allow controlled *trfA* expression may have a number of uses. For example, if a *cop* mutant of *trfA* is used, the copy number of the plasmid may be controlled by the inducer, indirectly making it possible to control the expression level of a gene controlled by another promoter. The vectors could also be used to insert transposons and inactivate specific genes by homologous recombination. Thus, the vector may be established in a host, the culture grown in the presence of inducer, and then plated onto selective plates in the absence of inducer. Only those cells where transposition or recombination has taken place will survive.

The ability to control *trfA* expression may be of interest from a safety point of view. The expression system makes it possible to eliminate the vector after production, since its existence is dependent on the particular inducer.

Modifications may also be introduced to any of the TOL-based regulatory functions. Thus, modifications, e.g. by introduction of point mutations including either by random or side-directed mutagenesis, may be made to the promoters *Pu* or *Pm* or to the regulatory genes *xylR* or *xylS*, for example to improve expression, alter the regulatory characteristics, or to extend the host range of the vectors, etc. For example, a mutant of the *Pm* promoter which exhibits down-regulation of expression, which might be useful in some circumstances, is reported

in Kessler et al., 1993, J. Mol. Biol. 230, 699-703. Conversely, mutations to enhance expression may also be made. Thus, for example, expression could be increased by expressing more XylS, as described for example by Kessler et al., 1994, J. Bacteriol., 176, 3171-3176. A number of modifications of the *xylS* gene have also been reported, for example the *xylS* mutant *xylS2tr6*, which exhibits an altered effector specificity, and can mediate a 3-8 fold higher level of transcription than can wild-type *xylS* at a wide range of temperatures (Ramos et al., supra), and the mutant gene *xylSarg41pro* (= *xylS*839), which causes a reduction in the basal transcription level from *Pm*, compared to wild type *xylS* (Michan et al., supra). All such modifications may be used according to the present invention.

It has also been found that the *xylR/xylS* genes may be inserted into the vectors in either orientation.

As mentioned above, the expression vectors of the invention may advantageously be used to express a desired gene within a broad range of host cells. It has surprisingly been found that high level and tightly controlled expression may be obtained across a broad range of hosts using the same vector system. This high level of expression maintained across a range of hosts is an unusual feature. In addition to the broad host range of the vectors, the *Pu* and *Pm* promoters give a very high induced to uninduced ratio, indicating that tight control of expression may be achieved. Especially, it has been observed that levels of expression from the *Pm* promoter are surprisingly high for different genes and for different hosts, as compared with *Pu* or other promoters. The use of a *Pm* promoter therefore represents a preferred aspect of the invention.

Transcription from the *Pu* and *Pm* promoters can be activated by different inducers, and different inducer compounds can lead to different levels of promoter activation (Ramos et al., 1990, J. Mol. Biol. 211, 373-

382). This property may also be used to fine-tune expression levels.

It may also be possible, further to modify expression levels by modifying culture conditions. Thus, the expression system may be improved by changing the growth condition of the host cell, e.g. temperature, culture medium composition and other culture conditions such as speed of agitation, vessel size etc. Such culture modifications are known in the art. It has been found, for example, that expression increases at lower temperature. It may further be possible also to modify expression from *Pu* by means of catabolite repression, for example by adding certain sugars, e.g. glucose to the growth medium during culture of the host cells.

The "genes" which may be expressed in the vectors of the invention include any desired or cloned genes including partial gene sequences, or any nucleotide sequence encoding a desired expression product, including fusion protein products, such as, for example, a desired gene sequence linked to a further nucleotide sequence encoding a further polypeptide such as β-galactosidase or glutathione-S-transferase. Such "fusion proteins" are well known in the art. The genes which are expressed from the vectors of the invention may thus include genes which are heterologous or homologous to the host cell.

The host range of the vectors is broad and includes a vast range of Gram-negative bacteria, as well as Gram-positive bacteria. Suitable Gram-negative bacteria include all enteric species, including, for example, *Escherichia* sp., *Salmonella*, *Klebsiella*, *Proteus* and *Yersinia*. and non-enteric bacteria including *Azotobacter* sp., *Pseudomonas* sp., *Xanthomonas* sp., *Caulobacter* sp, *Acinetobacter* sp., *Aeromonas* sp., *Agrobacterium* sp., *Alcaligenes* sp., *Bordatella* sp., *Haemophilus Influenzae*, *Methylophilus methylotrophus*, *Rhizobium* sp. and *Thiobacillus* sp. (see also Thomas and Helinski, supra). Gram-positive bacterial hosts which may be used include

Clavibacter sp.

Such transformed host cells are included within the scope of the present invention. A further aspect of the present invention thus includes a host cell containing an expression vector as hereinbefore defined.

Methods for introducing expression vectors into host cells and in particular methods of transformation of bacteria are well known in the art and widely described in the literature, including for example in Sambrook et al., (supra). Electroporation techniques are also well known and widely described.

In a still further aspect, the invention thus also provides a method of expressing a desired gene within a host cell, comprising introducing into said cell an expression vector as hereinbefore defined containing said desired gene, and culturing said cell under conditions in which said desired gene is expressed.

Advantageously, the desired gene may encode a desired polypeptide product and hence the invention also provides a method of preparing such a desired polypeptide product by culturing a host cell containing an expression vector of the invention into which the desired gene has been introduced, under conditions whereby said polypeptide is expressed, and recovering said polypeptide thus produced.

To express the desired genes, the expression vectors of the invention conveniently contain one or more sites for insertion of a cloned gene, e.g. one or more restriction sites, located downstream of the promoter region. Preferably, multiple, e.g. at least 2 or 3, up to 20 or more, such insertion sites are contained. Vectors containing multiple restriction sites have been constructed, containing eg. 20 unique sites in a polylinker. Suitable cloning sites for insertion of a desired gene are well known in the art and widely described in the literature, as are techniques for their construction and/or introduction into the vectors of the invention (see eg. Sambrook et

al., supra).

For ease of construction, appropriate cloning sites may be introduced in the form of a polylinker sequence, using nucleic acid manipulation techniques which are standard in the art. A range of suitable polylinker sequences are known in the art and may simplify the routine use of the expression vectors. Thus, for example a well-known polylinker/*lacZ'* region may be used, as described for example in the vectors of Ditta et al., 1985, Plasmid, 13, 149-153, simplifying standard cloning procedures and identification of plasmids with inserts, by using the blue/white selection technique based on *lacZ,* which is well-known in selection procedures.

A number of other features may also be included in the vectors of the invention. Thus, the vectors may include features which assist in plasmid transfer, such as the *oriT* function of RK2 plasmids, which facilitates conjugation and is useful in cases where transformation/ electroporation is inefficient, or if very high transfer frequencies are required.

Functions may also be introduced to stabilise the expression vectors, or to assist in their maintenance in a broad range of hosts. RK2 encodes two operons containing the *parDE* and *parcBA* genes, respectively, which are involved in the maintenance of RK2 plasmids or heterologous replicons in diverse bacterial hosts (Roberts et al., 1990, J. Bacteriol, 172, 6204-6216; Schmidhauser and Helinski, supra; Sia et al., 1995, J. Bacteriol, 117, 2789-2797; and Roberts et al., 1992, J. Bacteriol, 174, 8119-8132). *Par* functions or loci, including any of the *par* genes eg *parDE* may thus be introduced into the vectors of the invention.

Selectable markers are also usefully included in the vectors of the invention for example to facilitate the selection of transformants. A wide range of selectable markers are known in the art and described in the literature. Any of these may be used according to

the present invention and include for example the antibiotic resistance markers carried by the RK2 plasmids and their derivatives, or indeed any of the TOL plasmids or their derivatives, or any other plasmid. However, properties such as sugar utilisation, proteinase production or bacteriocin production or resistance may also be used as markers. The TOL plasmid *xylE* structural gene may also be used as a marker. This gene encodes the product C230 which may readily be detected qualitatively or assayed. Spraying a plate of bacterial colonies with catechol rapidly distinguishes $C230^+$ colonies since they turn yellow due to the accumulation of 2-hydroxy muconic semialdehyde, enabling transformants/transconjugants etc. rapidly to be identified, by the presence of *xylE* in the vectors.

Other features which may be included in the vectors include further regulatory and/or enhancer functions, for example transcriptional or translational control sequences such as start or stop codons, transcriptional initiators or terminators, ribosomal binding sites etc. Thus, for example, in vectors where *trfA* expression is not controlled, a transcriptional terminator, preferably a bidirectional terminator, may advantageously be positioned between the promoter and the *trfA* gene. In this way read-through transcription from the *trfA* gene into the *Pu/Pm* promoters may be prevented and transcription initiated at *Pu* or *Pm* should not affect *trfA* expression. It will however be appreciated that the use of transcriptional terminators has general applicability to avoid read-trough transcription of protein encoding portions of the vector, such as the *trfA* gene and the cloned gene of interest. Such functional elements are known in the art and a suitable transcriptional terminator is described in, for example, Fellay et al., 1987, Gene, 52, 147-154 and Frey and Krisch, 1985, Gene, 36, 143-150. As will be described in more detail in the Examples below, whilst TOL-based control elements such as start codons or ribosomal

binding sites etc. naturally associated with the *Pu/Pm* promoters may be used, alternative or additional such elements may also be introduced. Example 1 describes the preparation of an ATG expression vector, where sequences downstream of the ATG initiator were eliminated, permitting gene sequences to be inserted directly in this ATG site. A vector construct has also been created in which bases between the promoter and Shine-Dalgarno sequence are modified to create a new restriction site. Thus, the vector pJB653ATG of Example 1 has been modified in this fashion, making it possible to combine mutations in the Shine-Dalgarno sequence with mutations in the promoter.

Further modifications which may be made to the vectors, include size reduction by removal of unnecessary DNA from source or intermediate plasmids, removal of undesired restriction sites, addition of new restriction sites etc., which may be achieved by standard DNA manipulation techniques.

As mentioned above, the high levels of expression obtainable across a broad host range, make the expression vectors of the present invention particularly useful as tools for maximising and/or controlled expression of a desired gene product. Control of *trfA* expression permits a further means of regulating or controlling expression of a desired gene product. The vectors may also be used for expression studies and physiological analyses in bacteria, for example to analyse metabolic pathways, eg. determine rate limiting steps, conveniently also at intermediate or low expression levels, or for studies of plasmid transfer and dispersal in natural environments. The vectors of the invention may have particular utility as an environmental safety standard. The vectors of the invention, since they allow expression, and indeed in some cases replication of the vector, to be tightly controlled, are particularly safe from an environmental point of view. In particular, the *trfA* controlled

vectors would not be able to replicate in the natural environment, due to the absence of the inducer (except under certain cell growth temperatures - see Table 8), were they to escape into the environment, as a result of, for example, leakage of host cells from a fermentor. Thus, the vectors present in the escaped cells would eventually be eliminated as the escaped cells propagated, since the vectors would be unable to replicate, thereby also eliminating the inserted foreign gene from the environment.

The invention will now be described in more detail in the following Examples, with reference to the following drawings in which:

Figure 1 shows a map and the construction of general purpose broad host-range cloning vectors. Restriction sites relevant for the construction or use of the vectors are shown. Each step in the construction is indicated by an arrow. The restriction sites in the polylinker downstream the *lacZ* promoter is marked with ▾, and the sites are, in the counterclockwise direction; *Hind*III, *Sph*I, *Pst*I, *Sal*I/*Hinc*II/*Acc*I, *Xba*I, *Bam*HI, *Xma*I/*Sma*I, *Kpn*I, *Sac*I, and *Eco*RI. Sites in the polylinker that are not unique are indicated elsewhere on each vector. Note that the sites for *Nde*I and *Sfi*I are unique for all the vectors, except for pJB321.

Figure 2 presents graphs showing the broad host-range stabilization properties of the 0.8 kb *parDE* region in vector pJB321E. In various species: (A) *E.coli* DHSα; (B) *A. vinelandii*; (C) *P. aeruginosa*. Symbols: ■, pJB3E; °, pJB321E.

Figure 3 shows a map and the construction of broad host-range expression vectors pJB137 and pJB653. The sites in the polylinker (originally from pUC19) downstream of the promoters *Pm* and *Pu* are indicated. Other notations are as described in the legend to Figure 1. *Nde*I and *Sfi*I are unique in all the vectors, except for in the *parDE* derivatives pJB139 and pJB654 (Table 1).

<u>Figure 4</u> presents graphs showing Expression analysis of *celB* as a function of cell growth in *E.coli* PGM1. (A). Expression from pJB137celB (*Pu*). (B) Expression from pJB653celB (*Pm*). The basal expression level of *celB* from *Pm* is between 200 and 300 nmole/min/mg protein. (□), presence; (♦), absence of inducer dated at t = 0.

<u>Figure 5</u> shows amylose combination in *E.coli* PGM1 as a function of *celB* expression from pJB653celB.

<u>Figure 6</u> shows a map of pJB653ATG. pJB653ATG differs from pJB653 by lacking 275 bp downstream of the translation initiation ATG (underlined) and by the construction of an *Afl*III site at the initiating ATG by changing one base from C to A (marked with the symbol *). Note that pJB653ATG contains a unique *Pst*I site, in contrast to pJB653, which contains two such sites (Figure 3); RBS; ribosome binding site (32).

<u>Figure 7</u> presents graphs showing the expression of luc from pJB653ATG*luc* in (A) *E.coli* DH5α, (B) *X. campestris* and (C) *P. aeruginosa* as a function of cell growth in the presence (♦) and absence (□) of inducer. The basal expression levels of Luc from *Pm* in *E.coli*, *X. campestris* and *P. aeruginosa* are 4×10^6, 8×10^5 and 5.1×10^7 cpm, respectively (average values).
[a] The cpm values correspond to the activity in 10 μl cell culture at OD_{660}=0.3.

<u>Figure 8</u> shows an SDS-PAGE gel of samples of protein expressed in *E.Coli* DH5α from the "*CelB*" vectors of Example 2; lane 1: Molecular weight standard. lane 2: DH5α (pJB653ATG*celB*) induced. lane 3: DH5α (pJB653ATG*celB*) uninduced. lane 4: DH5α (pJB653ATG*celBcop271C*) induced. lane 5: DH5α (pJB653ATG*celBcop271C*) uninduced. lane 6: DH5α (pJB653ATG*celBcop251M*) induced. lane 7: DH5α (pJB653ATG*celBcop251M*) uninduced;

<u>Figure 9</u> shows a map of vector pJBSD1, as described in Example 4.

EXAMPLE 1

In this Example we describe the construction of a series of well characterized broad host-range multi-purpose cloning vectors based on the RK2 replicon. These vectors were used to develop tightly controlled gene expression systems. For this purpose we used the *Pu/Pm* promoters and the corresponding positive regulatory genes *xylR/xylS*, all originating from the TOL plasmid of Pseudomonas putida.

To characterise the functionality of the two promoters, we used the genes encoding the enzymes phosphoglucomutase (CelB) from Acetobacter xylinum (Fjærvik, et al., 1991, FEMS Microbial. Lett., 77, 325-330), and luciferase from the firefly *Photinus pyralis*. Amylose accumulation in *E.coli* was used as a model to study the intracellular effects of varying CelB expression, since *E.coli* cells lacking phosphoglucomutase (in contrast to wild type) accumulate amylose intracellularly when grown on maltose as carbon source (Adhya et al., 1971, J. Bacteriol., 108, 621-626).

The use of luciferase as a reporter was motivated by the fact that microorganisms generally do not naturally express this enzyme, in contrast to phosphoglucomutase.

Materials and Methods

Bacterial strains, plasmids and growth media.

The bacterial strains and plasmids used in this study are described in Table 1. *P. aeruginosa* and *E.coli* strains were grown in L-broth or on L-agar (Sambrook et al., supra). In the amylose accumulation experiments L-broth was supplemented with 1% maltose. The growth temperature was 30°C for *P. aeruginosa*. *E.coli* cells were grown at 37°C, except for the expression analysis of *celB* and *luc* transcribed from the

Pm/Pu promoters, where 30°C was used. *A. vinelandii* and *X. campestris* were grown at 30°C in Burk medium (Schmidhauser and Helinski, supra) and YM broth (Difco), respectively. Antibiotics were used at the following concentrations: ampicillin, 100 μg/ml (wild type *trfA*), 1 mg/ml (*cop271C*), or 2 mg/ml (*cop254D*); carbenicillin, 100 μg/ml; tetracycline, 15 μg/ml; chloramphenichol, 30 μg/ml; kanamycin, 50 μg/ml; streptomycin, 2 mg/ml.

Conjugative matings and electrotransformations.

Conjugative matings from *E.coli* to *P.aeruginosa* were performed on membranes and the mixtures were incubated on nonselective agar-medium at 30°C for 3 hours. S17.1 containing the relevant plasmids was used as donor strain. The mating mixture was incubated for 3 hours at 30°C and then plated on agar-medium containing carbenicillin and streptomycin. Plasmids were transferred to *A. vinelandii* and *X. campestris* by electrotransformation at a field strength of 12.5 kV/cm, as described for *E.coli* (Hanahan et al., 1991, Methods Enzymol, 204, 63-113) and the cells were then plated on agar-medium containing ampicillin.

DNA manipulations.

Plasmid DNA was prepared by the alkaline lysis protocol for *E.coli*, and all other standard techniques were performed according to Sambrook et al, supra. Transformations of *E.coli* were performed by the method of Chung et al., 1989, Proc. Natl. Acad. Sci., USA, 86, 2171-2175. DNA sequencing was performed by the dideoxy chain-termination method (Sanger et al., 1977, Proc. Natl. Acad. Sci., 74, 5463-5467). Cell growth at OD_{660} was monitored with a Beckman DU-65 (*celB* expression experiments) and a Shimadzu UV-160A spectrophotometer (*luc* expression experiments). For PCR amplification of the *luc* gene from pGEM*luc* the following primers were synthesized; 5'GATCCCCATGGAAGACGCCAA3' and 5'CGGAGGATCCCAATAGCTAAGAA3'. The primers contain a *Nco*I

and a *Bam*HI site, respectively. For PCR amplification of the 139 bp *Eco*RI/*Pst*I fragment, using pJB653 as template, the following primers were used; 5'AGGTGAATTCACATGTTCATGACTCCA3' (containing an *Eco*RI and an *Afl*III site), and 5'AGGGCTGCAGTGTCCGGTTTGA3' (containing a *Pst*I site).

Analysis of plasmid stability.

E.coli DH5α, *A. vinelandii* and *P. aeruginosa* containing pJB3E/pJB321E were grown under selection to stationary phase, diluted 100-fold in the same medium and then grown exponentially under selection. The stability assay was initiated by diluting the cells to 1 x 10^3 cells/ml in non-selective medium, followed by growth over night. Cultures were then again diluted and grown overnight in non-selective medium (as above), and this procedure was repeated until the total number of generations had reached 200-400, as indicated in the Results Section. After each dilution aliquots were plated on nonselective agar medium. The colonies were sprayed with 50 mM catechol to monitor the frequency of plasmid-containing cells (yellow colonies, Franklin et al., 1981, Proc. Natl. Acad. Sci, 78, 7458-7462).

The results were also double-checked by replica plating 100 colonies on agar-medium containing ampiciilin.

Expression studies and amylose measurements.

For CelB and Luc expression studies referring to Figure 4, Figure 7, and Table 3, cells were grown overnight in selective medium, diluted 100-fold in the same medium and then grown exponentially to OD_{660} = 0.1. Stimulation of *celB* and *luc* transcription from the *Pm* promoter was then induced by addition of *m*-toluic acid to 2 mM or 0.5 mM for *E.coli* and *X. campestris*, respectively. 0.5 mM IPTG was used for inducing *luc* expression from the *ptrc* promoter in p*Trc*99A*luc*. Cells containing pJB137*celB* were diluted again (2000-fold) and

grown to OD_{660} = 0.1. 3-Methylbenzylalcohol was then added to 3 mM for stimulating transcription from the *Pu* promoter. This extra step was included to eliminate background CelB remaining from stationary phase. Samples were removed at various time during growth for analysis of CelB or Luc activities.

For measurements of the CelB activities described in Table 2 cells were diluted 1000-fold after overnight growth and then grown to OD_{660} = 0.1 before addition of the inducer. For analysis of amylose accumulation cells were grown in selective medium overnight, diluted 200-fold, and then grown further to OD_{660} = 0.3-0.4. m-toluic acid was then added to 2 mM. Measurements of amylose accumulation (Brautaset et al., 1994, Microbiology, 140, 1183-1188) and CelB activities were performed 16 hours after addition of the inducer.

Preparation of cell-free extracts and measurements of phoshoglucomutase activities were performed as described by Fjærvik et al., (supra).

Measurements of luciferase activities were performed by using the Luciferase Assay System from Promega, and cell extracts were prepared from 90 µl cell culture, as described by the manufacturer. Samples were removed during growth and diluted or concentrated to OD_{660} = 0.3 before preparation of the extracts. 10 µl of the cell extracts was used for the quantitation of light intensity by a scintillation counter.

Results

Construction of general purpose broad host-range cloning vectors.

Figure 1 outlines the procedures involved in constructing a set of relatively small RK2-based vectors with different antibiotic resistance markers (pJB3, pJB3Cm6, pJB3Tc20, and pJB3Km1). Plasmid pFF1 was used as a starting point for all the constructs, and many of the steps in the construction procedure served to delete

unnecessary DNA sequences (size reduction), to eliminate undesired restriction endonuclease sites, or to create new such sites. One of the useful consequences of this is that the *Nde*I and *Sfi*I sites in *trf*A were kept unique. All vectors share in common a polylinker/*lacZ*' region. Most of the restriction endonuclease sites in the polylinker region are unique, and the exceptions are caused by the presence of some of these sites in antibiotic resistance marker genes. All vectors contain *oriT*.

The complete nucleotide sequences of the vectors were established by combining sequences previously reported in the literature and by sequencing many of the junction sites involved in the construction procedures. This greatly simplifies the routine use of the vectors, further improvements, and generation of more specialized derivatives.

Vector stability.

To improve plasmid stability for some hosts we inserted *parDE* into pJB3 generating pJB321, as shown in Figure 1. To simplify stability measurements the *xylE*' fragment from pJB109 was also inserted into the polylinker of pJB3 and in pJB321, generating plasmids pJB3*E* and pJB321*E*, respectively. The fragment was inserted in such an orientation that *xylE*' could be transcribed from the *lac* promoter in the vector. Figure 2 demonstrates the stabilizing effects of the *parDE* sequences in three different species. In *E.coli* the unmodified plasmid (pJB3*E*) is relatively stable, but in the presence of *parDE* (pJB321*E*) virtually no plasmid loss was observed (Figure 2A).

As can be seen from Figure 2B pJB321*E* is much more stable than pJB3*E*, illustrating the usefulness of this vector modification for certain hosts. In *Pseudomonas aeruginosa* the stability difference between the two plasmids was marginal (Figure 2C), but the frequency of plasmid loss is so low in both cases that for most

purposes practical problems should not be experienced.

Construction of broad host-range expression vectors.

Plasmid pJB7 was used as a starting point for the construction of expression vectors pJB137 and pJB653, containing the *Pu* and *Pm* promoters, respectively (Figure 3). In the first steps the genes encoding the positive regulators XylR and XylSArg41Pro were inserted. The mutant gene *xylSarg41pro* was used because it causes a reduction of the level from *Pm*, compared to wild type *xylS* (Michan et al., supra). The *Pu* and *Pm* promoters were then inserted, generating plasmids pJB134 and pJB64. The remaining steps up to the final constructs pJB137 and pJB653 served to fill in undesired restriction endonuclease sites, to create new sites, and to insert a bidirectional transcriptional terminator between the *Pu/Pm* promoters and the *trfA* gene. This terminator has previously been shown to function in a wide variety of Gram-negative species (Fellay et al., supra and Frey and Krisch, supra). To simplify the routine use of these expression vectors they contain a polylinker region downstream of the *Pu/Pm* promoters (Figure 3). In analogy to pJB321 (Figure 1) the *parDE* region was also inserted into each of the constructs, generating pJB139 and pJB654 (Table 1).

Expression of the *Acetobacter xylinum* phosphoglucomutase gene, *celB*, from the *Pu* and *Pm* promoters.

The 1.9 kb *Bam*HI *celB* fragment from pUC7*celB* was cloned in an orientation that allowed transcription of the gene from *Pu* in pJB137 and *Pm* in pJB653, generating pJB137*celB* and pJB653*celB*. The expression levels were then monitored as a function of cell growth (Figure 4A). As can be seen, the *Pu* promoter expresses very low levels of phosphoglucomutase in the absence of inducer as long as the cells are kept growing exponentially. The expression level in the presence of inducer is also low, but several fold higher than in uninduced cells.

As the cells enter stationary phase the expression levels in the uninduced and induced cells increases strongly, although the induced cells express much more of the enzyme.

Figure 4B shows the results of a corresponding expression study of pJB653*celB*, containing the *Pm* promoter. Expression from *Pm* does not seem to be affected significantly by the stage of growth but leakage and maximum expression are higher than for *Pu*. The results demonstrate that the leakage expression of this promoter is not growth phase dependent, and that the background level of expression is much higher than in exponentially growing cells containing pJB137*celB* (see legend to Figure 4). As subsequent experiments show, this backward expression is sufficiently low not to cause a problem. Moreover, if necessary to reduce leakage (uninduced) expression, a down mutant of the *Pm* promoter could be used (Kessler et al., supra). Stimulation of the *Pm* promoter resulted in much higher expression levels of CelB than from *Pu*. For unknown reasons the levels of phosphoglucomutase dropped significantly at prolonged incubation levels, in contrast to what was observed in the experiments with the *Pu* promoter.

The copy numbers of the vectors were increased by exchanging the *Sfi*I/*Nde*I fragment internally in the *trfA* gene. We have done this in pJB653*celB* and pJB137*celB* to analyse the copy number effects on *celB* expression (Table 2). For the *Pu* promoter in pJB137 the *cop*271C mutation leads to an increase in celB expression both in the absence and presence of inducer, and the magnitude of the increase is approximately proportional to the increase in copy number. (Haugen et al., 1992, supra). Surprisingly, however, when the copy number was increased further (about 20-fold) using *cop*254D (Haugen et al., 1992, supra) expression levels did not increase beyond the levels of *cop*271C. For the *Pm* promoter leakage expression increased strongly by introduction of

the *cop* mutations, while the *cop*254D mutant expressed even less phosphoglucomutase than *cop*271C.

The effects of the *cop*254D mutation on expression was rather puzzling, but we believe that the results may at least partly be caused by a poisoning effect on the cells mediated by the high copy number of *cop*254D (Haugan et al., 1995, supra). We observed directly that the PGM1 strain containing this mutant was somewhat inhibited in its growth rate, while such an effect was not observed in another *E.coli* strain, DH5α. As can be seen from Table 2, the expression levels of phosphoglucomutase for the *cop*254D/*Pm* combination were much higher in DH5α than in PGM1. These results thus strengthen the hypothesis that *cop*-mutant mediated cell poisoning effects may influence strongly the expression from *Pm*.

Use of pJB6S3*celB* for studies of effects of *celB* expression on amylose accumulation in *E.coli*.

Figure 5 demonstrates that when cells are grown on maltose as carbon source amylose accumulates in similar quantities as cellular protein in PGM1. In the presence of a low level of expression of *celB* (uninduced state of *Pm*) amylose accumulation is only slightly affected. In other words, the leakage synthesis is not sufficiently high to block amylose accumulation, illustrating that this promoter system can be used to analyse rate-limiting steps in metabolic pathways. In the presence of inducer amylose accumulation is strongly reduced, as expected, in response to the increase in the intracellular phosphoglucomutase level. However, we found it surprising that a significant accumulation still takes place in spite of the presence of very high levels of phosphoglucomutase. We believe that this effect is somehow the result of the particular biochemical properties of the *Acetobacter xylinum* phosphoglucomutase enzyme. This is clearly illustrated by the observation that the phosphoglucomcase positive

parent strain of PGM1 Hfr3000, does not accumulate measurable quantities of amylose (Brautaset, supra), even though the activity levels of the enzyme is as low as about 2% of the CelB activity under induced conditions (data not shown). This test system therefore seems to illustrate a case where a metabolic process can be modified by replacing an enzyme in a given host by a heterologous variant of the same enzyme.

Construction of an ATG vector and its use to study luciferase expression in *E.coli, X. campestris* and *P. aeruginosa*.

The DNA fragments containing the *Pu/Pm* promoters in pJB137/pJB653 both contain the ribosome-binding site. In addition, these fragments include for *Pm* the 5' terminal part of the first gene from the meta-cleavage pathway operon (Inouye et al., 1984, Gene, 29, 323-330) and for *Pu* the 5' terminal part of an ORF that has not been identified upstream of the first gene in the upper pathway operon (Harayama et al., 1989, J. Bacteriol. 171, 5048-5055; Inouye et al., 1984, Proc. Natl. Acad. Sci. 84, 1688-1691). This means that during expression of *celB* translation is probably first initiated at the natural signal sequences, and then reinitiated at the corresponding elements from *A. xylinum*. In order to create a more well-defined expression system we modified the region downstream of *Pm* in pJB653 such that the sequences downstream of the translation initiation ATG were eliminated, and new genes can be cloned directly in this ATG site after digesting the vector with *Afl*III (same cohesive ends as *Nco*I). *Afl*III was chosen since there is a *Nco*I site in the vector. The new vector was designated pJB653ATG (Figure 6). The *luc* gene from the firefly was then inserted at the ATG site, generating plasmid pJB653ATG*luc* (Table 1). This plasmid was then used to monitor *luc* expression in *E.coli, X. campestris* and *P. aeruginosa*. Our data based on expression of luc in pJB653ATG, show that it is possible to obtain more

than a 100-fold induction level in *X. campestris*. Figure 7A shows that the kinetics of activation in *E. coli* were similar to that of *celB* (Figure 4B), but the Luc activity was more stably maintained than the CelB activity upon prolonged incubation. Another difference is that the maximal ratio between the induced and uninduced state was significantly higher (between 300 and 400 fold) with pJB653ATG*luc* than with pJB653*celB* (between 50 and 100 fold). It is not clear whether this effect is somehow caused by the use of different reporter enzymes or by the changes introduced in the sequences downstream of the *Pm* promoter.

To quantitatively compare *luc* gene expression with some well-known expression vector we subcloned the *luc* gene at the ATG in the commercially available *E.coli* vector p*Trc*99A, generating p*Trc*99A*luc* (Table 1). These experiments showed that the Luc activity in such cells (after IPTG induction) was similar to the activities in induced cells containing pJB653ATG*luc*, while the induction ratio was much lower from pTrc99A (Table 3). The high levels of expression from the induced *Pm* promoter were unexpected, because the copy number of the RK2 replicon is much lower than that of p*Trc*99A, and also because p*trc* is known to be a very strong promoter. *Pm* has to our knowledge not been evaluated in this respect. To analyse these results further we inserted the *cop*271C mutation into the *trfA* gene of pJB653ATG*luc* and then repeated the expression experiments. The expression levels were much higher from this construct and exceeded the levels expressed from p*Trc*99A by a factor of seven. These data indicate that the *Pm* promoter may be useful for the purpose of maximizing gene expression.

To study the performance of pJB653ATG*luc* in a non-enteric host we transferred the plasmid to *X. campestris*, and measured *luc* expression in a similar way as in *E.coli*. Figure 7B demonstrates that as in *E.coli* *luc* expression is very low in uninduced cells, while the

activity increases more than 100-fold nine hours after induction. Figure 7C illustrates *luc* expression in *P. aeruginosa*, in which the maximum *luc* expression level was achieved 12 hours after induction, resulting in a 120-fold induction ratio. It can therefore be concluded that pJB653ATG*luc* has a broad potential for expression studies in bacteria.

Table 1. Bacterial strains and plasmids used in Example 1[a].

Bacterial strain or plasmid	Properties	Reference
Escherichia coli		
DH5α	*endA1 hsdR17 supE44 thi-1 λ gyrA96 relA1* Δ*lac*U169 (Φ*80dlacZ*ΔM15)	Bethesda Research Laboratories
S17.1	RP4 2-T::Mu-Km::Tn7 *pro res mod*$^+$	1
PGM1	*pgm* derivative of Hfr3000	2
Pseudomonas aeruginosa		
PA01161S	Spontaneous streptomycin resistant derivative of PA01161	3
Azotobacter vinelandii		
UW	Wild type	4
Xanthomonas campestris		
B100-152	Spontaneous exopolysaccharide mutant.	5
Plasmids		
RK2	60 kb broad-host-range plasmid originally isolated from *Klebsiella aerogenes* Ap^r.Km^r.Tc^r.	6
pFF1	RK2 minimal replicon Ap^r.Cm^r.5.9 kb.	7
pjB2	Derivative of pFF1 where the *Eco*R1, *Bgl*II, and *Sal*I sites were filled in by three steps. Ap^r.Cm^r.5.9 kb.	This work.
pUC19	*Col*E1 replicon Ap^r.2.7 kb.	8
pUC19-3	Derivative of PUC19 where the NdeI site was filled in (step 1) and the *Ssp*I and *Afl*III sites flanking the *lac* region were converted to *Nsi*I and *Bgl*II (steps 2 and 3, respectively). Ap^r.2.7 kb.	This work.
pJB5	Derivative of PJB2 where 0.5 kb of the upstream part of the Cm	This work

	resistance gene was deleted with *Pvu*II digestion, followed by insertion of a *Bgl*II linker at the same site (step 1). Two *Bam*H1 sites flanking *Pneo* were also filled in (step 2). Ap^r. 5.4 kb.	
pKH3	Derivative of pJB5 where 0.7 kb *Pst*I/*Bgl*II fragment was replaced with a 1.0 kb *Nsi*I/*Bgl*II fragment containing the polylinker and *lac* regions from pUC19-3. AP^r.5.7 kb.	This work
pJB7	Deletion derivative of pJB5 obtained by digestion with *Afl*III + *Eco*47III (0.4 kb, step 1) and *Not*I + partial *Acc*I digestion (0.5 kb, step 2). Ap^r. 4.5 kb.	This work
pJB3	Derivative of pJB7 where 1.5 kb *Bgl*II/*Sfi*I fragment was replaced with a 1.8 kb *Bgl*II/*Sfi*I fragment containing the polylinker and *lac* regions from pKH3. Ap^r. 4.8 kb.	This work
pRR120	pBluescript II SK(+) with 0.8 kb *parDE* region from RK2. Ap^r. 3.8 kb.	9
pJB9	Derivative of pRR120 where the polylinker sites between *Hind*III and *Sma*I, downstream of *parDE*, were deleted by digestion with *Hind*III (filled in) and *Sma*I. Ap^r. 3.8 kb.	This work
pJB10	Derivative of pJB9 where the *Kpn*I site upstream of *parDE* was converted to *Bgl*II. Ap^r. 3.8 kb.	This work
pHL12	Derivative of pJB9 where the *Bam*HI site downstream of *parDE* was filled in (step 1), and the *Kpn*I site upstream of *parDE* converted to *Xba*I (step 2). Ap^r. 3.8 kb.	This work
pJB313	Derivative of pJB3 where 0.8 kb *Bgl*II/	This work

	*Bam*HI fragment containing the *parDE* fragment from pJB10 was inserted into the *Bgl*II site. Ap^r. 5.6 kb.	
pJB321	Same as pJB313, except that the *parDE* fragment is in the opposite orientation.	This work
pαxylΩ	RSFIO1O replicon, Cm^r. 13.2 kb.	10
pUC7	ColE1 replicon. Ap^r 2.7 kb.	11
pJB107	Derivative of pUC7 where the promoterless *xylE* gene from pαxylEΩ was cloned as a 2.0 kb *Bam*HI fragment into pUC7 digested with the same enzyme. Ap^r. 4.7 kb.	This work
pJB109	Derivative of pJB107 where the two SacII sites flanking the *xylE* gene in pJB107 was converted to *Eco*RI sites (step 1). This 1.2 kb *Eco*RI fragment (here noted *xylE'*) was then cloned into pUC7 digested with *Eco*RI (step 2). Ap^r. 3.9 kb.	This work
pJB3E	Derivative of pJB3 where the 1.2 kb EcoRI *xylE'* fragment from pJB109 was cloned into the polylinker *Eco*RI site in PJB3. Ap^r. 6.0 kb.	This work
pJB313*E*	Derivative of pJB313 where the 1.2 kb *Eco*RI *xylE'* fragment from pJB109 was cloned into the polylinker *Eco*RI site in pJB313. Ap^r. 6.8 kb.	This work
pJB321*E*	Derivative of pJB321 where the 1.2 kb *Eco*RI *xylE'* fragment from pJB109 was cloned into the polylinker *Eco*RI site in pJB321. Ap^r. 6.8 kb.	This work
pSV16	RK2 replicon. AP^r. Km^r. 3.3 kb.	12
PJB3Km1	Derivative of pJB3 where the Km resistance gene of pSV16 was inserted into the *Bgl*II site as an 1.2 kb *Bam*HI fragment. Ap^r. Km^r. 6.1 kb.	This work
pJB3Km2	Same as pJB3Km1, except that the Km resistance gene was cloned in the	This work

	opposite orientation.	
pUC7Tc	Derivative of pUC7 where the Tc resistance gene of RK2 was cloned as a 2.3 kb blunt-ended *Stu*I/*Bgl*II fragment into the *Hinc*II site of pUC7. Ap^r. Tc^r. 5.0 kb.	This work
pJB3Tc20	Derivative of pJB3 where the Tc resistance gene from pUC7Tc was inserted as a 2.3 kb *Bam*HI fragment into the *Bgl*II site. AP^r. Tc^r. 7.1 kb.	This work
pJB3Tc19	Same as pJBTc20, except that the Tc resistance gene was cloned in the opposite orientation.	This work
pUC7Cm	Derivative of pUC7 where the Cm resistance gene was cloned as a 1.4 kb *Pst*I/*Hgi*AI blunt-ended fragment from pFF1 into the *Hinc*II site of pUC7. Ap^r. Cm^r. 4.1 kb.	This work
pJB3Cm6	Derivative of pJB3 where the Cm resistance gene of pUC7Cm was cloned as an 1.4 kb *Bam*HI fragment into the *Bgl*II site. Ap^r. Cm^r. 6.2 kb.	This work
pJB3Cm10	Same as pJB3Cm6, except that the Cm resistance gene was cloned in the opposite orientation.	This work
pJB8	Derivative of pJB7 where the *Nco*I site was converted to *Eco*RI. Ap^r. 4.5 kb.	This work
pERD839	RSF1010 replicon containing *xylS839*. Km^r. Sm^r. 14.7 kb	13
pJB86	Derivative of pJB8 where *xylS839* was cloned as a 1.7 kb *Bam*HI fragment from pERD839 into the *Bgl*II site. The *xylS839* gene is transcribed in the same direction as the *bla* and *trfA* gene. Ap^r. 6.2 kb.	This work
pERD21	RSF1010 replicon containing the *Pm* promoter. Km^r. 13.8kb.	14
pUC129	ColE1 replicon. Ap^r. 3.3 kb.	15

pJB103	pUC129 with *Pm* promoter cloned as an 0.6 kb *Eco*RI/*Pvu*II fragment from pERD21 into the EcoRI/EcoRV-digested vector. Ap^r. 3.9 kb.	This work
pJB64	Derivative of pJB86 where the *Pm* promoter was cloned as an 0.6 kb *Nsi*I/*Eco*RI fragment from pJB103 into pJB86 digested with *Pst*I and *Eco*RI. Ap^r. 6.8 kb.	This work
pJB651	Derivative of pJB64 where the orientation of *Pm* was reversed by digestion with *Kpn*I followed by religation (step 1). A series of restriction endonuclease sites upstream of *Pm* were eliminated by *Hind*III and *Eco*RI digestion (step 2), and downstream of *Pm* by *Sal*I and *Bam*HI digestion (step 3). The remaining *Kpn*I site downstream of *Pm* was converted to a *Hind*III site (step 4). Ap^r. 6.8 kb.	This work
pJFF350	ColE1 replicon containing transcriptional terminators of the Ω-Km transposable element. Km^r. 5.3 kb.	16
pJBI7	Derivative of pUC19 where the *Xba*I site in the polylinker was filled in (step 1), and the polylinker *Pst*I site was converted to *Xba*I (step 2). Ap^r. 2.7 kb.	This work
p7B1725	The 3.6 kb blunt-ended *Hind*III fragment containing the Ω transcriptional terminators and the Km resistance gene from pJFF350 was cloned into the *Hinc*II site of pJB17 (step 1). The Km and *ori* region (3.0 kb) from pBR322 was deleted by *Sty*I digestion (step 2). Ap^r. 3.3 kb.	This work
pJB1726	The *Xba*I site in pJB1725 was converted to a *Hind*III site. Ap^r. 3.3 kb.	This work
pJB652	Derivative of pJB651 where the Ω transcriptional terminators of pJB1726	This work

	were cloned as an 0.6 kb *Hind*III/*Eco*RI fragment into pJB651 digested with the same enzymes. Ap^r. 7.4 kb.	
pJB653	Derivative of pJB652 where the *Pst*I fragment containing the *Pm* promoter was cloned in the opposite direction by digesting pJB652 with *Pst*I followed by religation. This step was necessary since DNA sequencing showed that *Pm* was in the incorrect orientation in pJB652. Ap^r. 7.4 kb. (It should be noted that although *xylS* was cloned from pERD839, sequencing data indicates that pJB653 contains wild-type *xylS* - this is reflected in Figure 6.)	This work
pJB654	The *Xba*I site upstream *parDE* in pJB139 and the *Bbs*I site upstream *xylS839* in pJB653 were filled in (step 1). Originally, there were two *Xba*I sites and two *Bbs*I sites flanking *parDE* and *xylS839*, respectively. The 3.0 kb *Sfi*I/*Bbs*I (*Bbs*I made blunt) fragment of pJB653 was replaced with the 3.8 kb *Sfi*I/*Xba*I (*Xba*I made blunt) *parDE* containing fragment from pJB139. Ap^r. 8.2 kb.	
pTS174	pACYC184 replicon, carries *xylR*. Cm^r.	17
pJB101	Derivative of pUC7 where a 2.4 kb *xylR*-containing *Hpa*I fragment was cloned into the polylinker *Hinc*II site of pUC7. Ap^r. 5.1 kb	This work
pJB13	Derivative of pJB8 where the *xylR* gene of pJB101 was cloned as a 2.4 kb *Bam*HI fragment into the *Bgl*II site of pJB8. The *xylR* gene is transcribed in the same direction as the *bla* and *trfA* gene. Ap^r. 6.9 kb.	This work
pRD579	R1 replicon, carries the *Pu* promoter. Cb^r.	18

pUC18	ColE1 replicon. Ap^r. 2.7 kb.	8
pJB105	Derivative of pUC18 where the *Pu* promoter was cloned as an 0.3 kb *Eco*RI/*Bam*HI fragment from pRD579 into pUC18 digested with the same enzymes. Ap^r. 3.0 kb.	This work
pJB134	Derivative of pJB13 where the *Pu* promoter was cloned as an 0.4 kb *Eco*RI/*Pst*I fragment from pJB105 into pJB13 digested with the same enzymes. Ap^r. 7.0 kb.	This work
pJB136	Derivative of pJB134 where the *Eco*RI site upstream of the *Pu* promoter was filled in (step 1), and the *Bam*Hl site downstream of *Pu* was converted to *Eco*RI (step 2). Ap^r. 7.0 kb.	This work
pJB137	Derivative of pJB136 where the Ω transcriptional terminators from pJB1725 were cloned as an 0.6 kb *Eco*RI/*Xba*I fragment into pJB136 digested with the same enzymes. Ap^r. 7.6 kb.	This work
pJB139	Derivative of pJB137 where the *Xba*I site was filled in (step 1), and the *Tth*I site converted to *Xba*I (step 2). The *parDE* fragment from pHL12 was inserted into the *Xba*I site as a 0.8 kb *Xba*I fragment (step 3). The *parDE* gene is transcribed counterclockwise to the *xylR* gene. Ap^r. 8.4 kb.	This work
pTB16	ColE1 replicon. Ap^r. 4.3 kb.	19
pUC7*celB*	Derivative of pUC7 where the 1.9 kb blunt-ended SphHI celB fragment from pTB16 was cloned onto the *Hinc*II site of pUC7. Ap^r. 4.6 kb.	This work
pJB137*celB*	Derivative of pJB137 where the 1.9 kb *Bam*HI *celB* fragment from pUC7*celB* was cloned in pJB137 digested with the same enzyme. *celB* is transcribed	This work

	from the *Pu* promoter. Ap^r. 9.5 kb.	
pJB653*celB*	Derivative of pJB653 where the 1.9 kb *Bam*HI *celB* fragment from pUC7*celB* was cloned in pJB653 digested with the same enzyme. *celB* is transcribed from the Pm promoter. Ap^r. 9.3 kb.	This work
pFF1*cop254D*	pFF1 containing the *cop254D* mutation. Ap^r. Cm^r. 5.9 kb.	3
pFF1*cop271C*	pFF1 containing the *cop271C* mutation. Ap^r. Cm^r. 5.9 kb.	20
pJB137*celBcop254D*	Derivative of pJB137*celB* where the 0.6 kb *Nde*I/*Sfi*I fragment was replaced with the 0.6 kb *Nde*I/*Sfi*I fragment from pFFI*cop254D* containing the *cop254D* mutation. Ap^r. 9.5 kb.	This work
pJB137*celBcop271C*	Derivative of pJB137*celB* where the 0.6 kb *Nde*I/*Sfi*I fragment was replaced with the 0.6 kb *Nde*I/*Sfi*I fragment from pFF1*cop271C* containing the *cop271C* mutation. Ap^r. 9,. kb.	This work
pJB653*celBcop254D*	Derivative of pJB653*celB* where the 0.6 kb *Nde*I/*Sfi*I fragment was replaced with the 0.6 kb *Nde*I/*Sfi*I fragment from pFF1*cop254D* containing the *cop254D* mutation. Ap^r. 9.3 kb.	This work
pJB653*celBcop271C*	Derivative of pJB653*celB* where the 0.6 kb *Nde*I/*Sfi*I fragment was replaced with the 0.6 kb *Nde*I/*Sfi*I fragment from pFF1*cop271C* containing the *cop271C* mutation. Ap^r. 9.3 kb.	This work
pGEM-*luc*	pGEM-*luc* contains the *luc* gene encoding firefly luciferase. Ap^r. 4,9 kb.	Promega
p*Trc*99A	Expression vector containing the trc promoter. ColE1 replicon. Ap^r. 4.2 kb.	Pharmacia LKB Biotechnology
p*Trc*99A*luc*	Derivative of p*Trc*99A where the *luc* gene from pGEM-*luc* was cloned as a 1.7 kb *Nco*I/*Bam*HI fragment amplified by PCR into p*Trc*99A digested with the same.	This work

	enzymes. Ap^r. 5.9 kb.	
pJB653ATG	ATG expression vector. A derivative of pJB653 where the 413 bp *Eco*R1/*Pst*I fragment containing the *Pm promoter* is replaced with a 139 bp *Eco*RI/*Pst*I fragment containing *Pm* and *an Afl*III site. Ap^r. 7.2 kb.	This work
pJB653ATG*luc*	The *luc* gene from pGEM-*luc* was cloned as a 1.7 kb *Nco*I/*Bam*HI fragment into the *Afl*III/*Bam*HI site of pJB653ATG. Ap^r. 8.9 kb.	This work
pJB653ATG*luccop271C*	Derivative of pJB653ATG*luc* where the 1.5 kb *Bam*HI/*Sfi*I fragment was replaced with the 1.5 kb *Bam*HI/*Sfi*I fragment from pJB653*celBcop271C*. Ap^r. 8.9 kb.	This work

[a] Ap^r, ampicillin resistance; Cm^r, chloramphenicol resistance; Km^r, kanamycin resistance; Tc^r, tetracycline resistance; Cb^r, carbenicillin resistance.

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TABLE 2. CelB activities as a function of plasmid copy number in *E.coli*

Strain	CelB activity (nmole/min/mg protein)		
	t = 0 hours	uninduced[a]	induced[a]
PGM1 (pJB137*celB*)	10	450	1300
PGM1 (pJB137*celBcop271C*)	90	1400	3500
PGM1 (pJB137*celBcop254D*)	100	1400	2800
PGM1 (pJB653*celB*)	200	250	13000
PGM1 (pJB653*celBcop271C*)	4000	2000	30000
PGM1 (pJB653*celBcop254D*)	4000	1000	9000
DH5α (pJB653*celB*)	450	360	15300
DH5α (pJB653*celBcop254D*)	15300	14000	59100

[a] Cells were harvested 4 (pJB653*celB*) or 6 (pJB137*celB*) hours after induction.

TABLE 3. Luc activity as a function of plasmid copy number in *E.coli* DH5α

plasmid	# hours[a]	Luc activity ($cpm \times 10^6$) uninduced	induced	ratio
pJB653ATG*luc*	3	1.1	170	155
pJB653ATG*luc*	5	1.7	670	394
pJB653ATG*luccop271C*	3	7.5	1400	187
pJB653ATG*luccop271C*	5	14	3500	250
pTrc99A*luc*	3	48	540	11
pTrc99A*luc*	5	34	520	15

[a] t = 0 hours at induction

EXAMPLE 2

Materials and Methods

In this Example the expression from *Pm*, of three genes, *luc*, *celB*, and *cat*, encoding chloramphenicol acetyltranferase (CAT) was compared in *E.coli*, *X. campestris* and *P. Aeruginosa*. The *trf*A mutation designated *cop251M* has been previously isolated by Durland et al., 1990 (supra) and has also independently been isolated by us. This copy up mutant was cloned into the expression vector pJB653ATG (see Example 1), using techniques as described in Example 1, generating pJB653ATG*cop251M*. Further following the procedures of Example 1, the *luc* gene was inserted into pJB653ATG*cop251M*, generating pJB653ATG*luccop251M*.

As a comparison, plasmid pT7-7(1.9) was constructed, in which *celB* was cloned into pT7-7 (United States Biochemical Corporation (USB), Cleveland, Ohio,

USA; Tabor and Richardson, 1985, Proc. Natl. Acad. Sci. USA, 262, 1074-1078) as a 1.9 kb *Nde*I/*Pst*I PCR fragment into the *Nde*I and *Pst*I sites of pT7-7. The fragment for cloning was prepared by PCR techniques using standard methods. An *Nde*I site at the ATG in *CelB* was created in a PCR reaction using appropriately modified primers.

Plasmids pJB653ATG*luc*, pJB653ATG*luccop271C*, p*Trc*99A*luc*, pJB653ATG*celB*, pJB653ATG*celBcop271C* were as prepared in Example 1.

The vector pJB653ATG*cat* was constructed as follows: *cat* was cloned as a 662 bp *Nco*I *Bam*HI fragment from pCat3Basic (from Promega) into *Afl*III/*Bam*HI in pJB653ATG (obtained according to Example 1). First, the *Xba*I site downstream *cat* in pCat3Basic was converted to a *Bam*HI site by the use of a *Bam*HI linker (NEB) after making the *Xba*I site blunt by Klenow. The comparative vector pTrc99A*cat* was constructed as follows: *cat* was cloned as a *Nco*I/*Bam*HI fragment (as above) into *Nco*I/*Bam*HI in pTrc99A.

Expression studies

All strains were grown as described in Example 1. Transcription from *Pm* was induced by 2 mM or 0.5 mM *m*-toluic acid in *E.coli* and *X. campestris*, respectively. 0.25 mM IPTG was used for induction of expression from the p*Trc* promoter. The strain containing pT7-7(1.9) was grown in LB medium + ampicillin + kanamycin overnight at 30°C. The cells were diluted 50-fold and grown further for 3 hours. Cells containing the *Pm* vectors were diluted 100-fold. *celB* expression was induced by heat at 42°C for 30 minutes and the cells were grown for another 1.5 hours at 30°C. Preparation of cell-free extracts and measurements of phosphoglucomutase and luciferase activities are described in Example 1. The preparation of cell extracts for chloramphenicol acetyltransferase activities were performed as described by Sambrook et al., 1989, supra (a modified version); 1) Cells were harvested from 1 ml culture by centrifugation

at 12000g, 1 minute at 4°C. 2) The cell pellets were resuspended in 100μl of freshly prepared 1 mg/ml egg white lysozyme, 20% sucrose, 30 mM Tris-Cl pH 8.0, 1 mM EDTA pH 8.0. On ice for 10 minutes. 3) Lysis was completed by freezing/thawing in liquid N_2/37°C (2x). In case of deacetylases in the cell extract, the extracts were incubated at 65° for 10 minutes, followed by centrifugation at 12000g for 10 minutes. CAT activity was measured according to the protocol of the *Quan*-T-CAT assay system from Amersham Life Science.

Results

The results are presented in Tables 4, 5 and 6. It will be seen that in addition to *luc* and *celB*, the *cat* gene may also be expressed from the expression vectors of the invention, at significant levels of expression (Table 5). These data indicate that the *Pm* promoter is a strong system for expression. Table 4 shows that *luc* expression was even better in *Pseudomonas* as compared with *E.coli*. Luc is better expressed from a wild-type *trfA* vector in *Pseudomonas*, than from *cop* mutants in *E.coli*, suggesting that there is a potential for further improvement in expression in *Pseudomonas*. In the case of *cat* expression, expression in *Pseudomonas* with wild-type *trfA* is better than in *E.coli*, but not with *cop* mutants.

TABLE 4. Measurements of Luc activity in *E.coli*, *P. aeruginosa* and *X. campestris*

Strain/plasmid	# hours[a]	Luc activity cpm x 10^6 uninduced	induced
E.coli			
DH5α			
pJB653ATGluc	5	2	560
pJB653ATG*luccop271C*	5	35	3400
pJB653ATG*luccop251M*	5	94	5000
p*Trc*99A*luc*	5	34	520
Xanthomonas campestris			
B100-152			
pJB653ATG*luc*	21	0.6	240
Pseudomonas aeruginosa			
PAO1161.S			
pJB653ATG*luc*	12	51	6200

[a] t = 0 hours at induction

TABLE 5. Measurements of CAT activity in *E.coli*, *P. aeruginosa* and *X. campestris*

Strain/plasmid	# hours[a]	CAT activity dpm x 10^6 uninduced	CAT activity dpm x 10^6 induced
E.coli			
DH5α			
pJB653ATG*cat*	5	0.14	32
pJB653ATG*catcop271C*	5	2	62
pJB653ATGcatcop251CM	5	7	270
p*Trc*99A*cat*	5	130	140
Xanthomonas campestris			
B100-152			
pJB653ATG*cat*	16	0.03	0.85
Pseudomonas aeruginosa			
PAO1161S			
pJB653ATG*cat*	12	0.79	78

[a] t = 0 hours at induction

TABLE 6. Measurements of CelB activity in *E.coli*

Strain/plasmid	# hours[a]	CelB activity[b] (nmole/min/mg protein) uninduced	induced
E.coli			
DH5α			
pJB653ATG*celB*[d]	5	1920	138000
pJB653ATG*celBcop271C*	5	12850	329000
pJB653ATGcelBcop251M	5	62300	455500
PGM1			
pT7-7(1.9)		nd[c]	445000

[a] t = 0 hours at induction

[b] Preparation of cell extracts: 10 ml cell culture was resuspended in 3 ml 40 mM imidazol-HCl pH 7.4 before sonication expr. For pT7-7(1.9), 5 ml cell culture was resuspended in 3 ml 40 mM imidazol-HCl pH 7.4.

[c] nd = not determined in this experiment, but previous results have shown that the uninduced state is approximately 50% lower than induced state.

[d] The pJB653ATG vector used for expression of *celB* is not the same as used for *luc* and *cat* expression analysis. The vector used for *celB* expression has an *Nde*I site in the ATG start site and not an *Afl*III site. The vector suitable for *celB* expression may be produced as follows: The *Pst*I site upstream of the polylinker in pJB653*Nde*I-A (see Table 7) was made blunt, and the *Hind*III/*Sfi*I fragment of pJB653*Nde*I-A was replaced by the 848-bp *Hind*III/*Sfi*I fragment containing the *trfA* gene from pTB*trfA*2. The *Pst*I site (originally from the *Hind*III/*Sfi*I fragment of pTB*trfA*2) was made blunt. Ap^r. 6.8kb. pTB*trfA*2 was produced as follows: *trfA* was cloned as a 1.2 kb *Pst*I/*Eco*RI fragment from pRD110-34

(Table 7) into the same sites in pALTER-1 (Table 7). The *Nde*I site in the *trfA* gene was eliminated by site-specific mutagenesis. Tc^r. 6.9 kb.

EXAMPLE 3

An SDS-PAGE (8% polyacrylamide) was performed on samples of protein expressed in *E.coli* DH5α from the "*celB*" vectors of Example 2, using standard procedures as described in Sambrook et al, supra, as follows:

Sample	Protein conc. (mg/ml)	#μl extract loaded on gel
Wild-type *trfA* (wt) 0mM	0.65	8.2
Wild-type *trfA* (wt) 2mM	0.53	10
cop271C 0mM	0.72	7.4
cop271C 2mM	0.53	10
cop251M 0mM	0.76	6.9
cop251M 2mM	0.56	9.5

The concentrations 0mM and 2mM refer to the inducer (see Example 2).

The results are shown in Figure 8, which show *celB* expression as protein, rather than activity, from the various "CelB" vectors of Example 2.

EXAMPLE 4

The vector pJBSD1 was constructed in which in the vector pJB653ATG of Example 1, the location of *trfA* was altered such that it was deleted from its original location in pJB653ATG and placed downstream of the *Pm* promoter. pJBSD1 is shown in Figure 9, and details of its construction are summarised in tabular form in Table 7 below, with reference to the following source plasmids:-

<u>Characteristics and References of the plasmids used in the construction of pJBSD1</u>

1.	pJB653ATG	-	ATG expression vector (see Example 1).
2.	pRD110 - 34	-	pBR322 replicon where an EcoRI/Pst fragment was substituted with the *trfA* gene from plasmid RD2. Durland et al., J. Bacteriol, 172, 3759-3867 (1990).
3.	pALTER®-1	-	Mutagenesis vector used in the Altered sites® II in vitro mutagenesis system. From Promega.
4.	pSELECT™-1	-	Mutagenesis vector used in the Altered sites™ in vitro mutagenesis system. From Promega.
5.	pTB16	-	A plasmid carrying celB gene encoding phosphoglucomutase. Brautaset et al, Microbiology 140, 1183-1188(1994).

TABLE 7: Description of the plasmids used in the construction of pJBSD1

1.	pJB653ATG	-	ATG expression vector (see Example 1), Ap^r, 6.8 kb.
2.	pJB653*Nde*I-A	-	Derivative of pJB653ATG in which the *Afl*III site was converted to a *Nde*I site by replacing the 143 bp *Pst*I/*Eco*RI fragment of pJB653ATG with the *Pst*I/*Eco*RI PCR fragment containing the *Nde*I site, Ap^r, 6.8 kb.
3.	pJB653*Nde*I-B	-	Derivative of pJB653*Nde*I-A in which the *Pst*I site upstream of the *Pm* promoter has been filled in, Ap^r, 6.8 kb.
4.	pRD110-34	-	ColE1 replicon where *Eco*R1-*Pst*1 fragment of pBR322 was substituted

with the *trfA* gene from plasmid RK2, Tc^r, 4.8 kb. (Durland et al., J. Bacteriol., 172, 3759-3867 (1990).)

5. pALTER-1 - Mutagenesis vector used in the Altered sites II in vitro mutagenesis system, Tc^r, 5.7 kb. From Promega.

6. pALTER*trfA*-1 - *trfA* was cloned as a 1.2 kb *Pst*I/*Eco*RI fragment from pRD110-34 into the same sites in pALTER-1, Tc^r, 6.9 kb.

7. pALTER*trfA*-*Nde*I - Derivative of pALTER*trfA*-1 in which the *Nde*I site in the *trfA* gene was eliminated by site specific mutagenesis, Tc^r, 6.9 kb.

8. pJB653*Nde*IC2 - Derivative of pJB653*Nde*I-B in which the 1.2-kb *Hind*III/*Sfi*I fragment was replaced with the 1.2 kb *Hind*III/*Sfi*I fragment from pALTER*trfA*-*Nde*I, Ap^r, 6.8 kb.

9. pJB653*Nde*IC2b - Derivative of pJB653*Nde*IC2 in which the *Pst*I site has been filled in, Ap^r, 6.8 kb.

10. pSELECT -1 - Mutagenesis vector used in the Altered sites *in vitro* mutagenesis system, Tc^r, 5.7 kb. From Promega.

11. pTB16 - A ColE1 replicon carrying *celB* gene encoding phosphoglucomutase, Ap^r, 4.3 kb. (Brautaset et al., Microbiology 140, 1183-1188 (1994).)

12. pSEL(1.9)B - The *celB* gene from pTB6 was cloned as a 1.9 kb *Sph*I fragment into the same site in pSELECT-1. *Nde*I site was made at the start codon of *celB* by site directed mutagenesis, Tc^r, 7.6 kb.

13. pJB653*Nde*IC2b*CelB* - Derivative of pJB653*Nde*IC2b in which the *celB* gene from pSEL(1.9)B was cloned as a 1.9 kb *Nde*I/*Bam*HI fragment into the same sites of pJB653*Nde*IC2b, Ap^r, 8.7 kb.
14. pJB653*Nde*IC2b*trfA* - Derivative of pJB653*Nde*IC2b*CelB* in which the 1.9 kb *Nde*I/*Pst*I fragment containing *celB* gene was replaced with a 1.2 kb *Mse*I/*Pst*I fragment containing the *trfA* gene, Ap^r, 8 kb.
15. pJBSD1 - Derivative of pJB653*Nde*IC2b*trfA* in which the *trfA* gene downstream of *Pneo* promoter was deleted with *Pvu*II/*Hind*III digestion followed by filling in and religation of the vector part, Ap^r, 6.6 kb.

Ap^r = ampicillin resistance
Tc^r = tetracycline resistance

PJBSD1 was transferred to *E.coli* DH5α as described in Example 1, and the cells were grown in LB medium overnight at 30°C in the presence of 1 mM toluate and 0.1 mg/ml of ampicillin. Some plates were incubated at 23°C for 2 days. Cells were then diluted and plated on LB medium containing the ampicillin and toluate concentrations indicated in Table 8, at approximately 100 cells per plate. The plates were incubated at the temperatures indicated in Table 8 and the results are shown in Table 8. + means that colonies appeared after overnight incubation, while - means no growth.

Reading the data in the Table 8 horizontally, it will be seen that replication is controlled by the inducer level. It appears that slightly less inducer is required as the temperature is lowered; at 23°C the plasmids appear to replicate even in the absence of inducer. The reason for this could be that *Pm* is better

expressed, that the beta-lactamase is better expressed or more active, that the functionality of TrfA increases somewhat, or that the plasmid copy number increases slightly. Possibly, more TrfA is made at low temperatures or less is required for replication. Such TrfA expression at low temperatures could be dealt with by introducing mutations in *Pm* or its Shine-Dalgarno sequence, such that *trfA* expression is reduced. If Table 8 is read vertically, it will be noted that the ampicillin resistance level is affected by the inducer concentration, even at a fixed temperature. This must mean that as inducer concentrations are being lowered, *trfA* expression becomes reduced. This first leads to copy number reductions (reduced ampicillin tolerance) and then (no inducer) to total block of replication (no growth even at low ampicillin concentrations). The properties of vector pJBSD7 are thus remarkable and unique.

Table 8

Control of pJBSD1 replication by the externally added inducer m-Toluic acid

Ampicillin concentration in mg/ml	Toluic acid concentration in mM	23°C					30°C					37°C					42°C				
		0.0	0.1	0.5	1.0	2.0	0.0	0.1	0.5	1.0	2.0	0.0	0.1	0.5	1.0	2.0	0.0	0.1	0.5	1.0	2.0
0.1		+	+	+	+	+	-	+	+	+	+	-	-	+	+	+	-	-	+	+	+
0.2		+	+	+	+	+	-	+	+	+	+	-	-	+	+	+	-	-	+	+	+
0.4		+	+	+	+	+	-	+	+	+	+	-	-	+	+	+	-	-	-	+	+
0.6		-	-	-	+	+	-	-	-	+	+	-	-	-	+	+	-	-	-	-	+
0.8		-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

CLAIMS

1. An expression vector comprising an RK2 minimum replicon together with an expression cassette comprising the regulatory functions of a TOL plasmid.

2. An expression vector as claimed in claim 1, comprising a RK2 minimum replicon together with a promoter *Pm* and/or *Pu* and a corresponding regulatory gene *xylS* and/or *xylR* as derived from a TOL plasmid.

3. An expression vector as claimed in claim 1 or claim 2, wherein, in said RK2 minimum replicon, the *trfA* gene is a copy-up (*cop*) mutant.

4. An expression vector as claimed in any one of claims 1 to 3, wherein said RK2 minimum replicon carries mutations in the *trfA* gene that are temperature-sensitive for replication.

5. An expression vector as claimed in any one of claims 1 to 4 wherein the *trfA* gene is under control of the *Pm* and/or *Pu* promoter.

6. An expression vector as claimed in any one of claims 1 to 5, comprising *Pm* and a gene selected from native *xylS*, *xylS2tr6*, and *xylSarg41pro*, or any mutant thereof.

7. An expression vector as claimed in any one of claims 1 to 6, comprising a polylinker/*lacZ'* region.

8. An expression vector as claimed in any one of claims 1 to 7, comprising an RK2-derived *OriT*.

9. An expression vector as claimed in any one of claims 1 to 8, comprising a stabilisation function.

10. An expression vector as claimed in claim 10, wherein said stabilisation function comprises *par* loci.

11. An expression vector as claimed in any one of claims 1 to 10, further comprising one or more further regulatory and/or enhancer functions.

12. A host cell containing an expression vector as defined in any one of claims 1 to 11.

13. Method of expressing a desired gene within a host cell, comprising introducing into said cell an expression vector as defined in any one of claims 1 to 11 containing said desired gene, and culturing said cell under conditions in which said desired gene is expressed.

14. A method of preparing a desired polypeptide product by culturing a host cell containing an expression vector as defined in any one of claims 1 to 12 into which the desired gene has been introduced, under conditions whereby said polypeptide is expressed, and recovering said polypeptide thus produced.

15. A host cell or method as claimed in any one of claims 12 to 14, wherein said host cell is selected from *Escherichia* sp., *Salmonella*, *Klebsiella*, *Proteus*, *Yersinia*, *Azotobacter* sp., *Pseudomonas* sp., *Xanthomonas* sp., *Caulobacter* sp., *Acinetobacter* sp., *Aeromonas* sp., *Agrobacterium* sp., *Alcaligenes* sp., *Bordatella* sp., *Haemophilus Influenzae*, *Methylophilus methylotrophus*, *Rhizobium* sp., *Thiobacillus* sp., and *Clavibacter* sp.

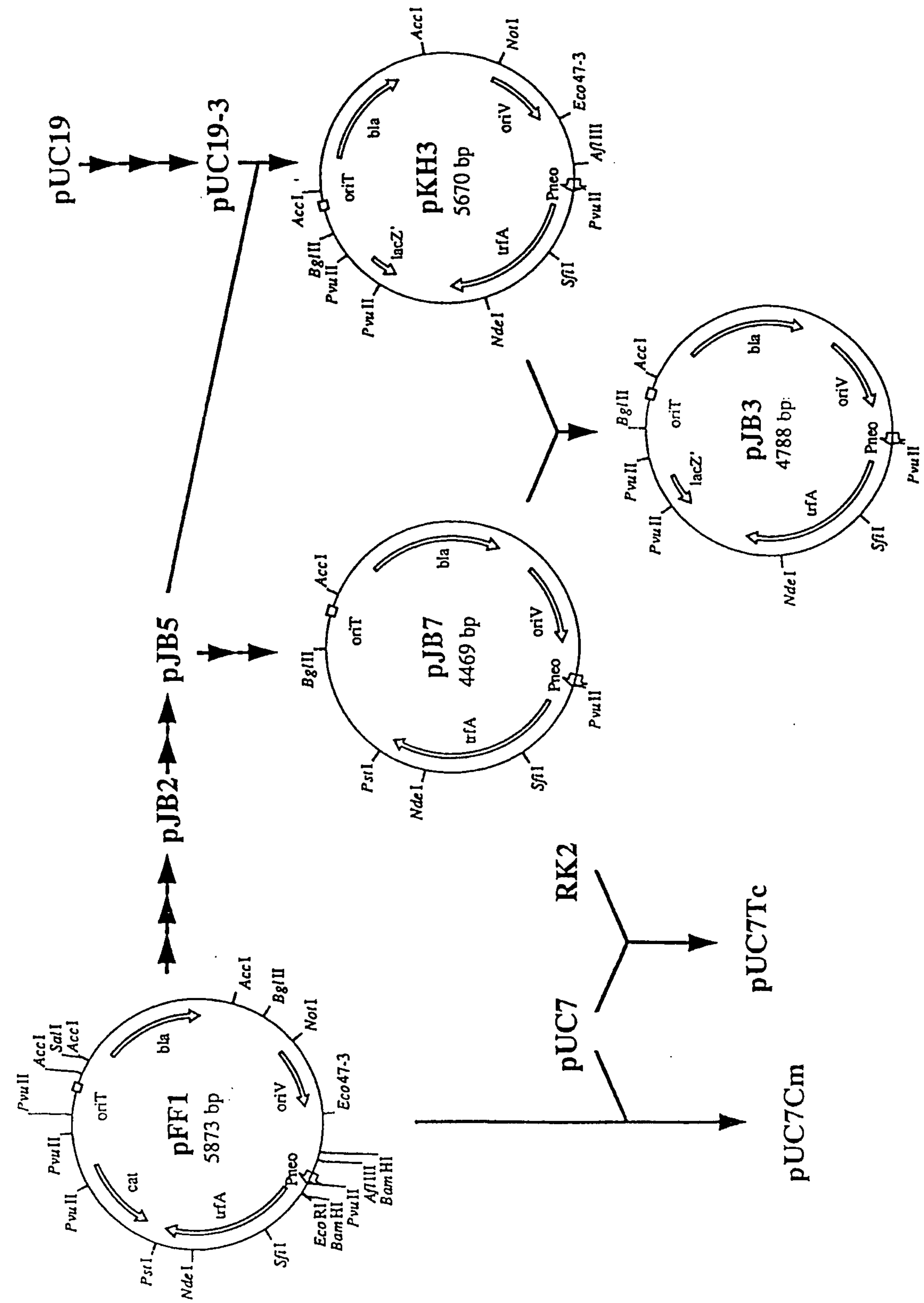

pUC19
pUC19-3
pKH3
5670 bp
pJB3
4788 bp
pJB5
pJB2
pJB7
4469 bp
pFF1
5873 bp
RK2
pUC7
pUC7Tc
pUC7Cm
bla
oriV
oriT
urfA
Pneo
lacZ'
cat
AccI
NotI
Eco47-3
AflIII
PvuII
SfiI
NdeI
BglII
PstI
SalI
EcoRI
BamHI

Figure 1

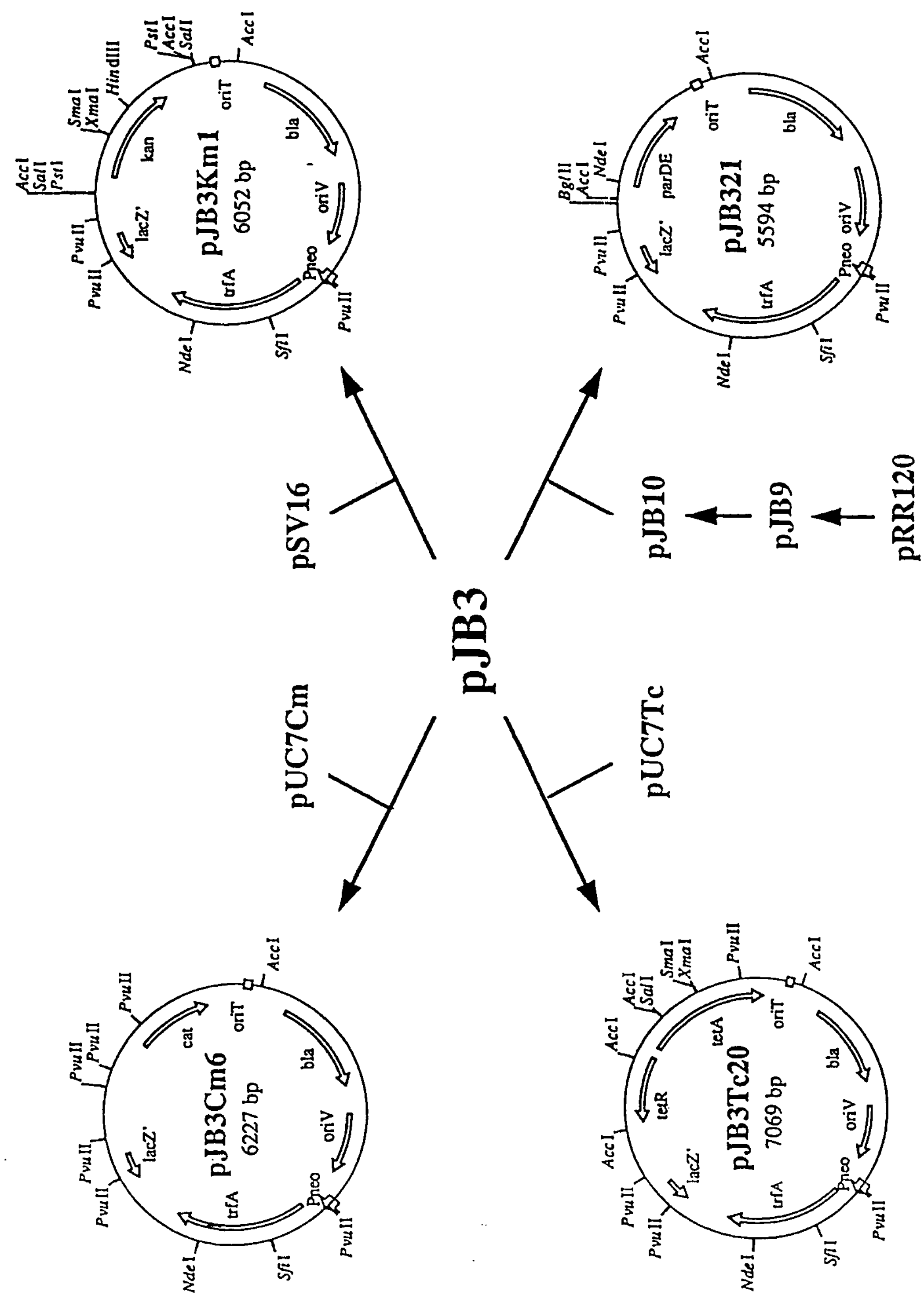


Figure 1 (continued)

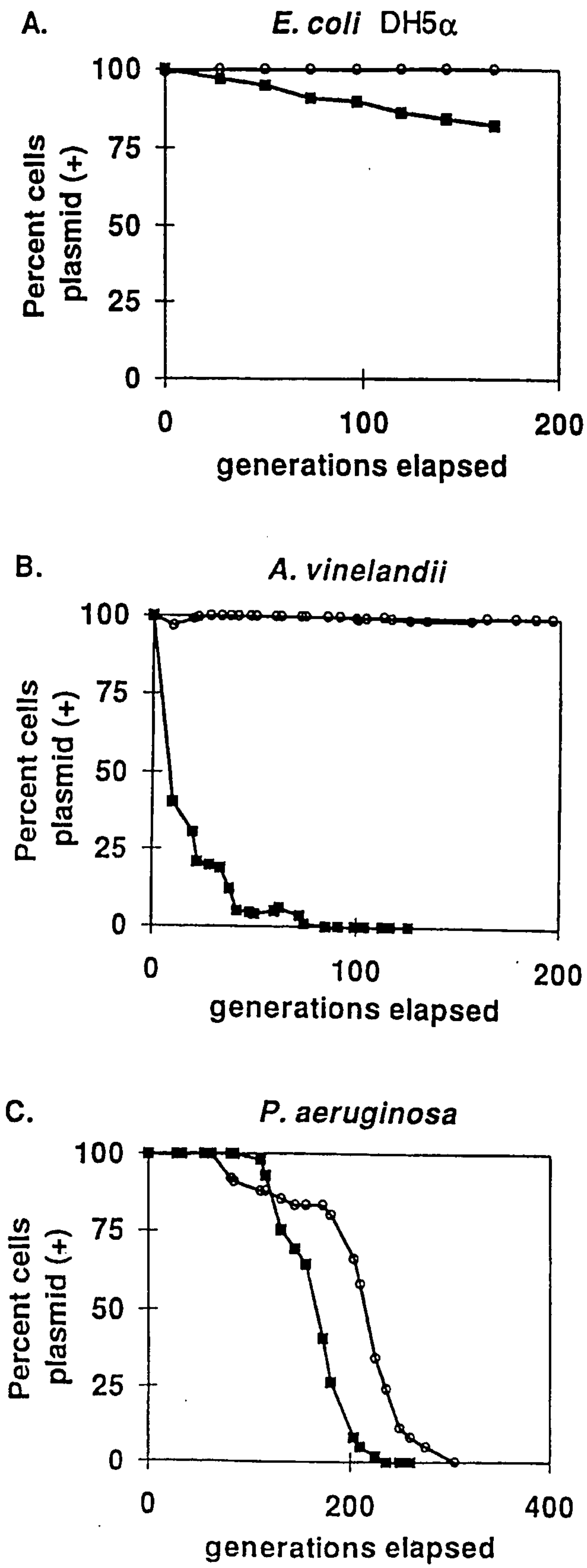
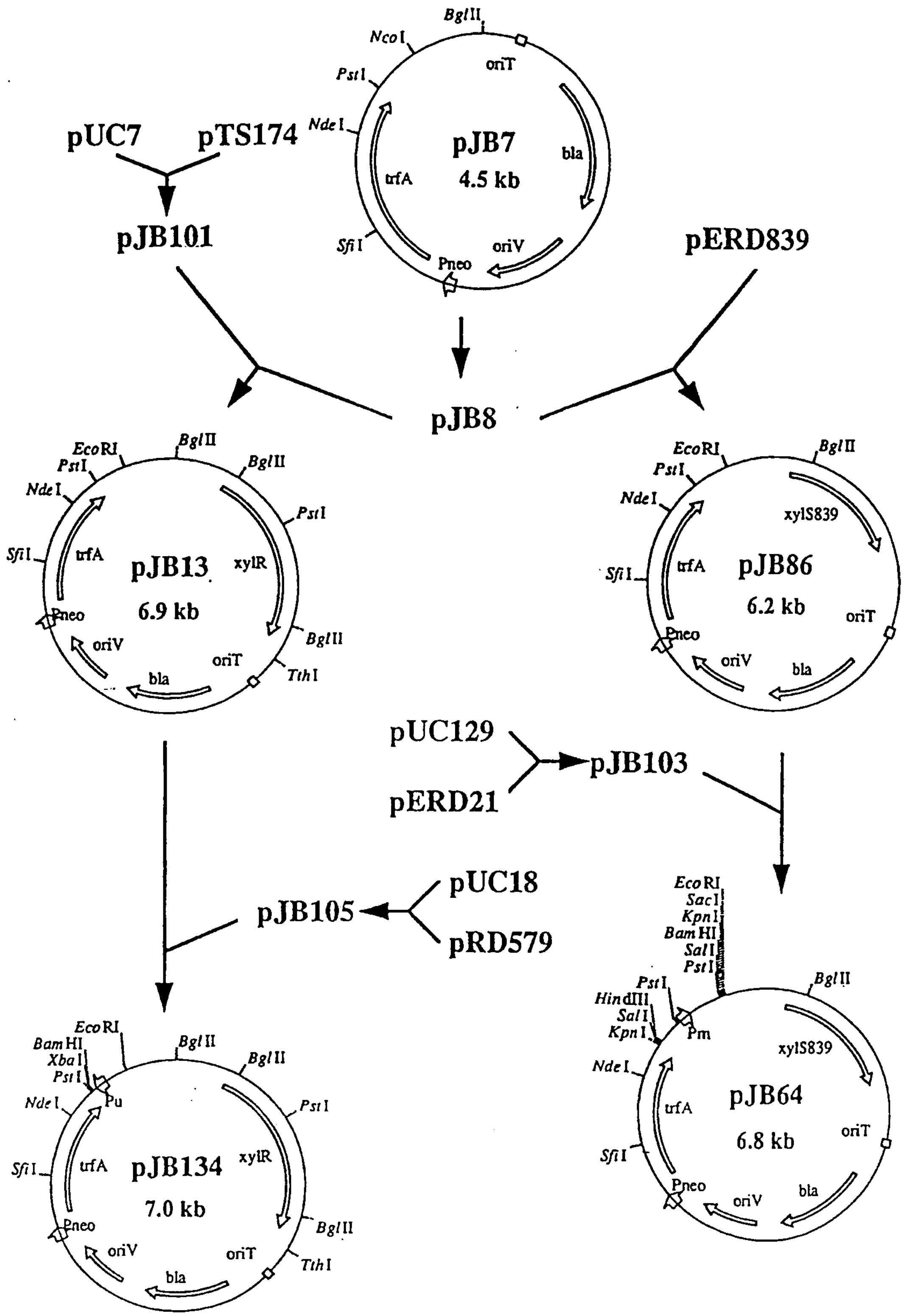

A.
E. coli DH5α
Percent cells plasmid (+)
100
75
50
25
0
0
100
200
generations elapsed
B.
A. vinelandii
Percent cells plasmid (+)
100
75
50
25
0
0
100
200
generations elapsed
C.
P. aeruginosa
Percent cells plasmid (+)
100
75
50
25
0
0
200
400
generations elapsed

Figure 2



pUC7
pTS174
pJB101
BglII
NcoI
PstI
NdeI
SfiI
oriT
bla
trfA
pJB7
4.5 kb
oriV
Pneo
pERD839
pJB8
EcoRI
PstI
NdeI
SfiI
BglII
BglII
PstI
trfA
pJB13
6.9 kb
xylR
Pneo
oriV
bla
oriT
BglII
TthI
EcoRI
PstI
NdeI
SfiI
BglII
xylS839
trfA
pJB86
6.2 kb
oriT
Pneo
oriV
bla
pUC129
pERD21
pJB103
pUC18
pRD579
pJB105
EcoRI
BamHI
XbaI
PstI
NdeI
SfiI
BglII
BglII
PstI
Pu
trfA
pJB134
7.0 kb
xylR
Pneo
oriV
bla
oriT
BglII
TthI
EcoRI
SacI
KpnI
BamHI
SalI
PstI
PstI
HindIII
SalI
KpnI
NdeI
SfiI
BglII
Pm
xylS839
trfA
pJB64
6.8 kb
oriT
Pneo
oriV
bla

Figure 3

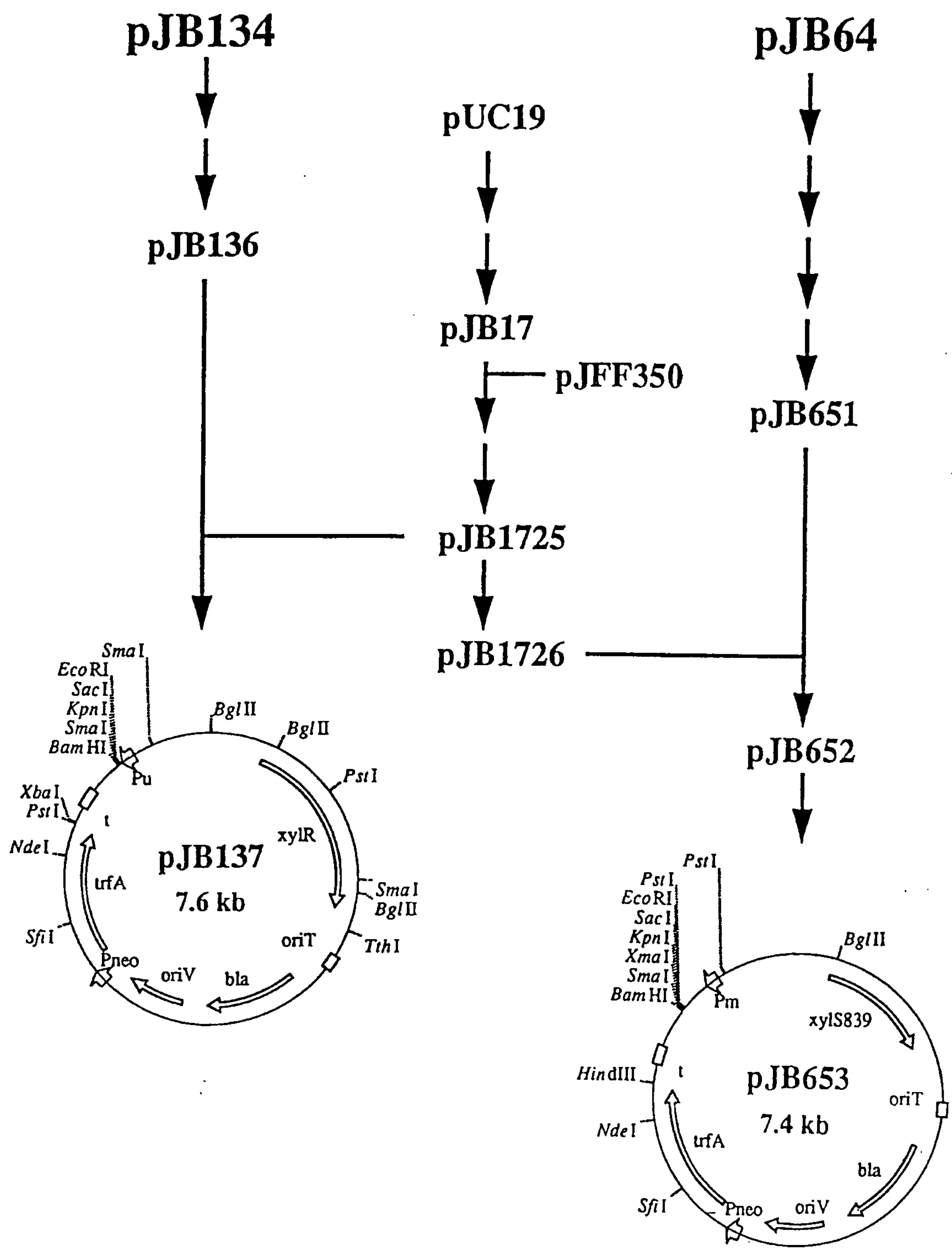
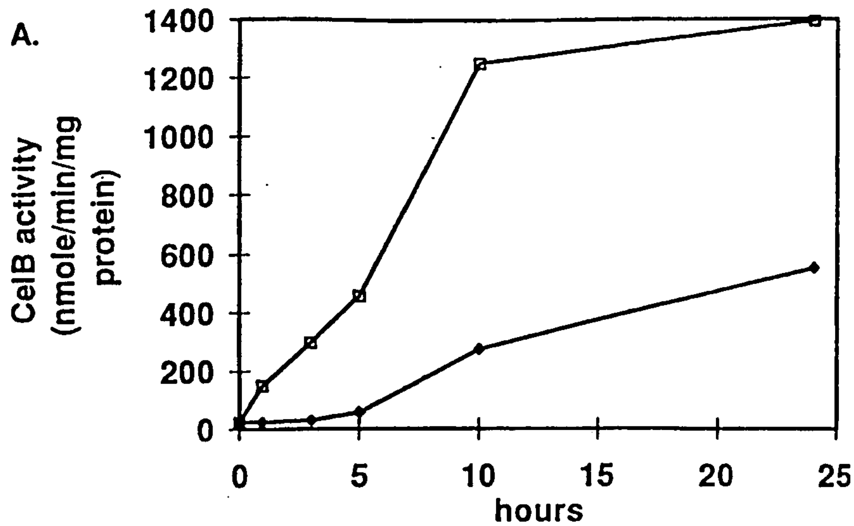


Figure 3 (continued)

A.


CelB activity (nmole/min/mg protein)
1400
1200
1000
800
600
400
200
0
0
5
10
15
20
25
hours

B.

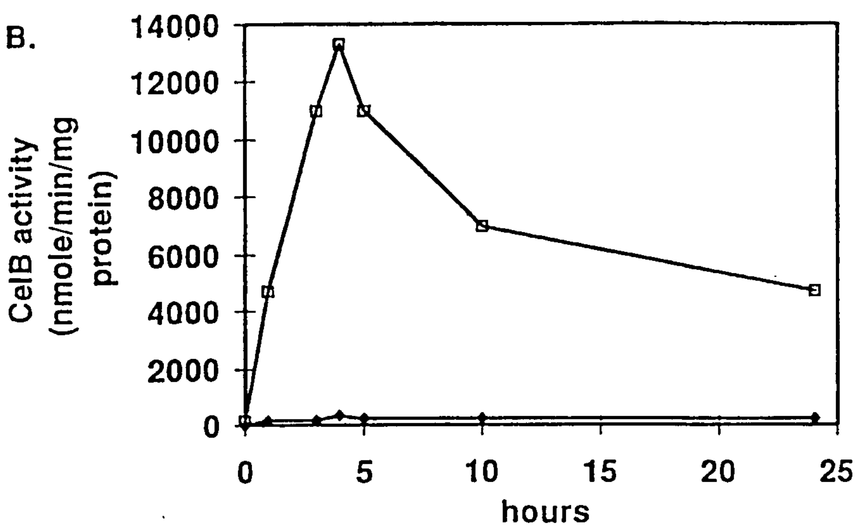

CelB activity (nmole/min/mg protein)
14000
12000
10000
8000
6000
4000
2000
0
0
5
10
15
20
25
hours

Figure 4

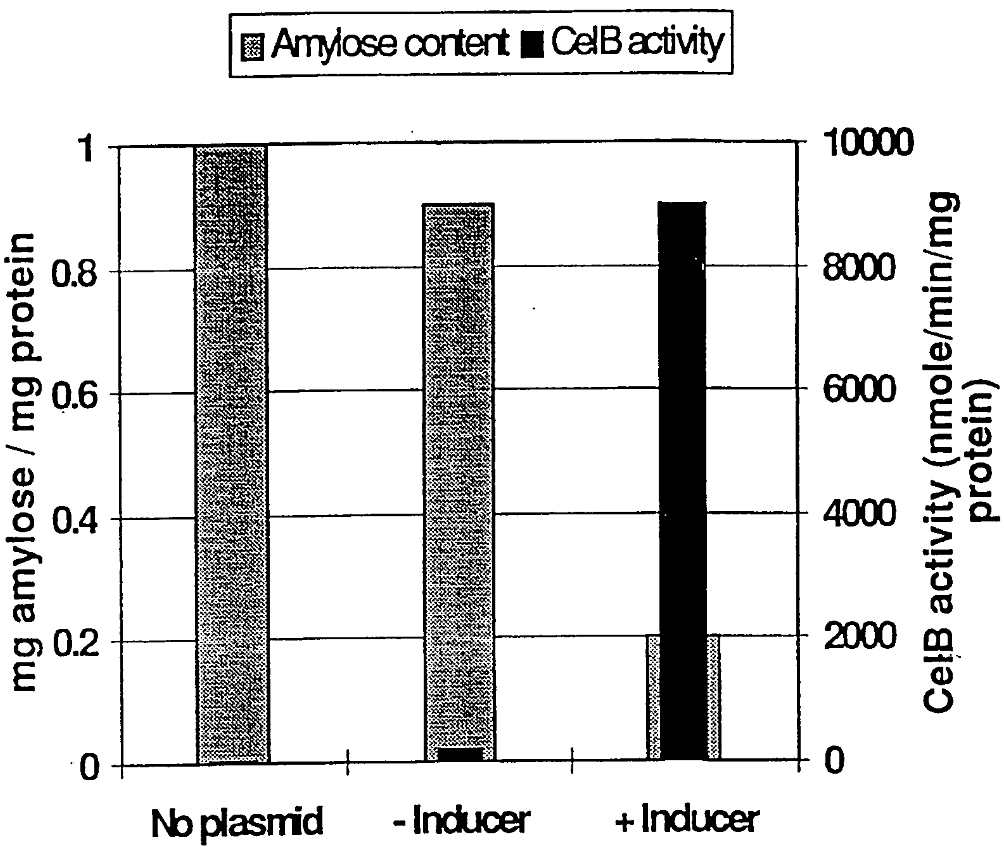
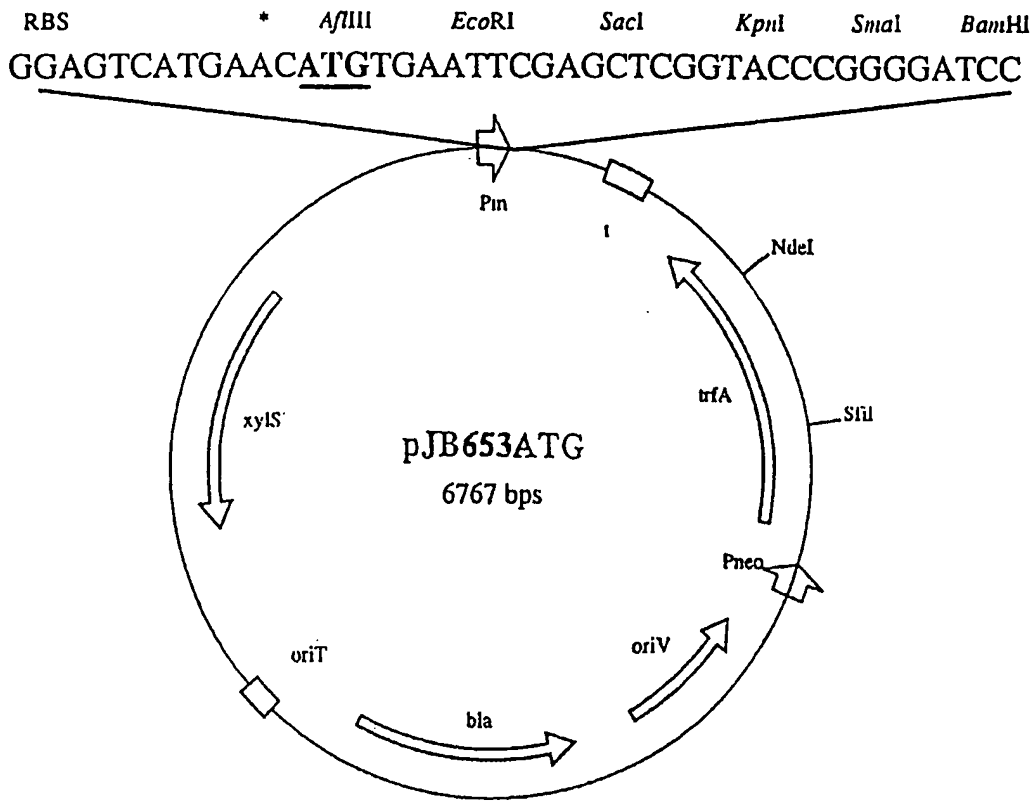
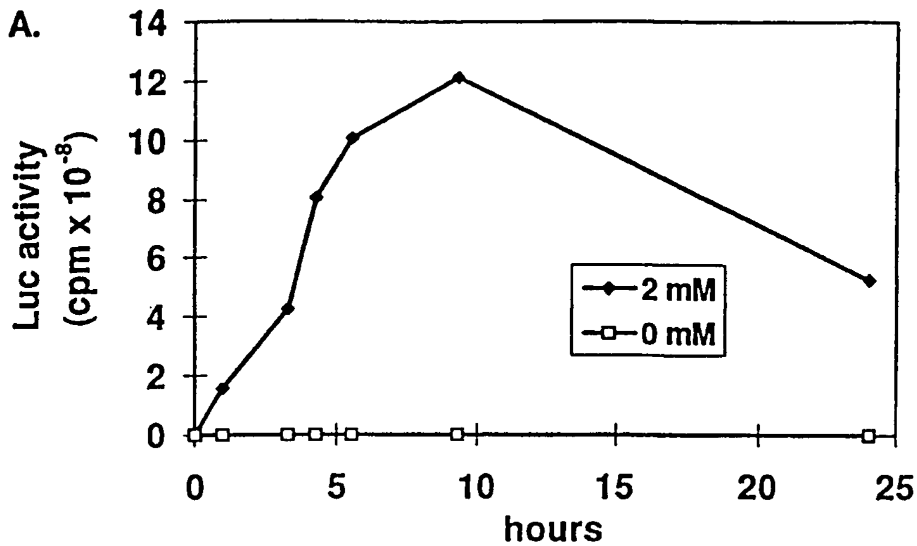

Amylose content
CelB activity
mg amylose / mg protein
CelB activity (nmole/min/mg protein)
1
0.8
0.6
0.4
0.2
0
10000
8000
6000
4000
2000
0
No plasmid
- Inducer
+ Inducer

Figure 5



RBS
*
AflIII
EcoRI
SacI
KpnI
SmaI
BamHI
GGAGTCATGAACATGTGAATTCGAGCTCGGTACCCGGGGATCC
Pm
t
NdeI
trfA
SfiI
xylS
pJB653ATG
6767 bps
Pneo
oriV
oriT
bla

Figure 6


A.
Luc activity
(cpm x 10^{-8})
14
12
10
8
6
4
2
0
0
5
10
15
20
25
hours
2 mM
0 mM

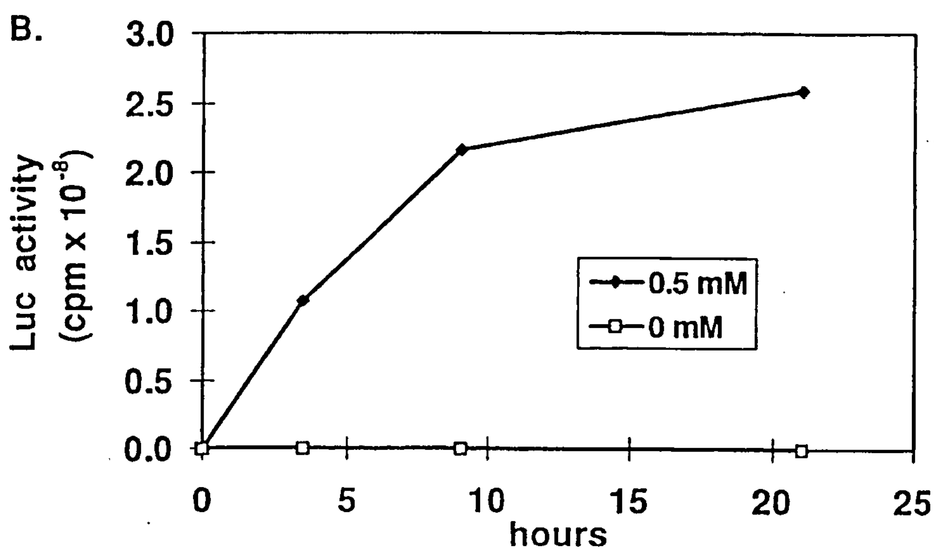

B.
Luc activity
(cpm x 10^{-8})
3.0
2.5
2.0
1.5
1.0
0.5
0.0
0
5
10
15
20
25
hours
0.5 mM
0 mM

Figure 7

C.

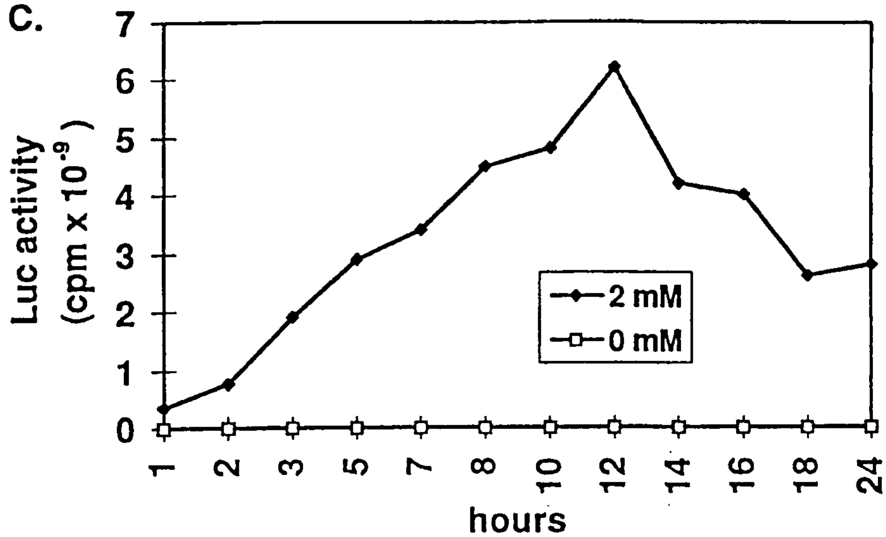


Figure 7 (continued)

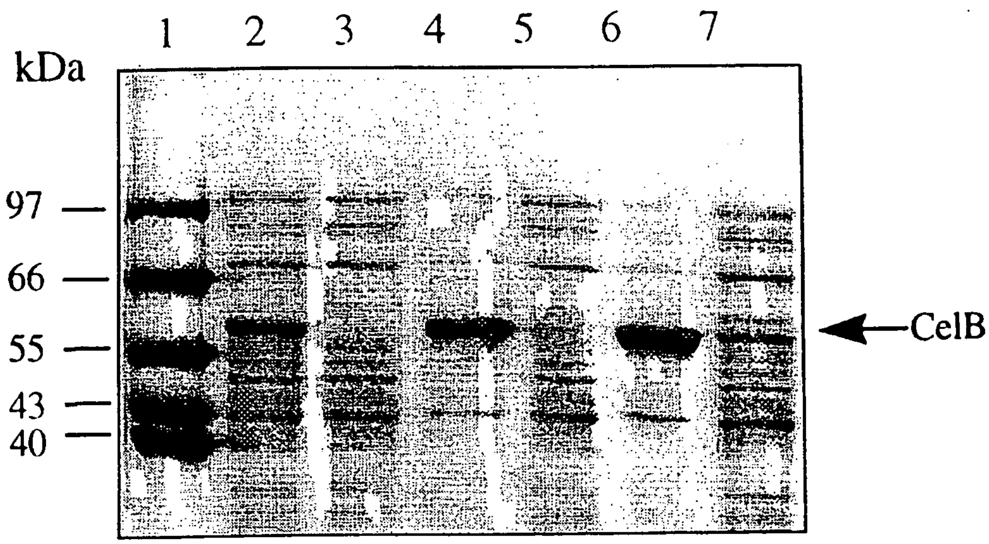


FIG. 8.

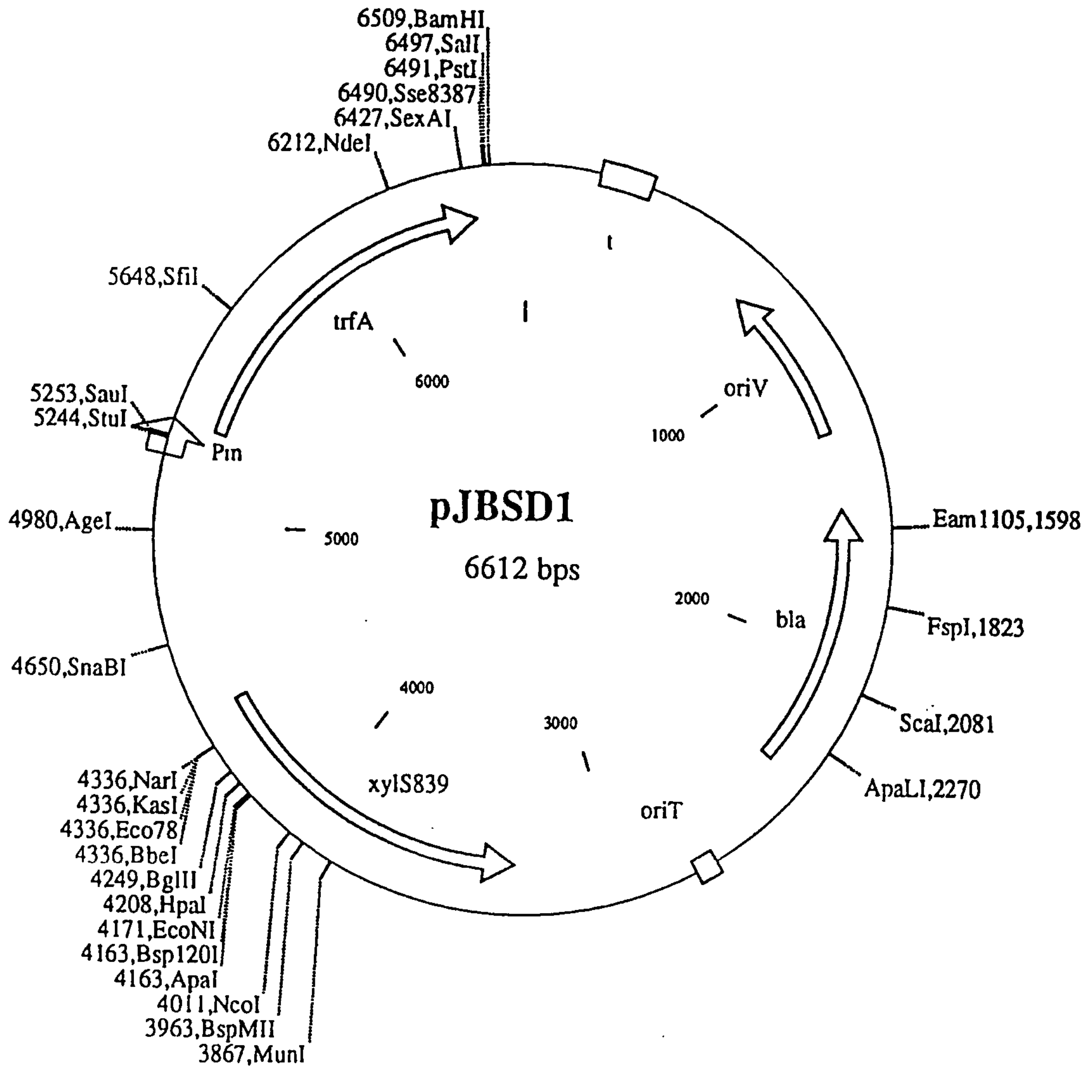

6509,BamHI
6497,SalI
6491,PstI
6490,Sse8387
6427,SexAI
6212,NdeI
5648,SfiI
5253,SauI
5244,StuI
4980,AgeI
4650,SnaBI
4336,NarI
4336,KasI
4336,Eco78
4336,BbeI
4249,BglII
4208,HpaI
4171,EcoNI
4163,Bsp120I
4163,ApaI
4011,NcoI
3963,BspMII
3867,MunI
Eam1105,1598
FspI,1823
ScaI,2081
ApaLI,2270
trfA
Pm
oriV
bla
oriT
xylS839
t
pJBSD1
6612 bps
1000
2000
3000
4000
5000
6000

Figure 9

INTERNATIONAL SEARCH REPORT

International Application No
PCT/GB 97/02323

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C12N15/63 C12N15/68 C12N15/69 C12N15/70 C12N15/74
C12N1/21 //C12N15/53,C12N15/54,C12N15/61,(C12N1/21,C12R1:19,
C12R1:385,C12R1:64)

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 C12N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	WO 89 09823 A (UNIV BRUNEL ;3I PLC (GB)) 19 October 1989 see the whole document ---	1,2,5,6, 9-15
Y	WO 91 16439 A (RHONE POULENC BIOCHIMIE) 31 October 1991 see the whole document ---	1,2,5,6, 9-15
Y	EP 0 443 063 A (HENKEL RESEARCH CORP) 28 August 1991 see page 3, line 22 - line 26 see page 3, line 50 - line 52 see page 4, line 8 - line 12 see page 22, line 1 - line 23 --- -/--	1,2,5,6, 9-15

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

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Date of the actual completion of the international search	Date of mailing of the international search report
21 November 1997	29/12/1997
Name and mailing address of the ISA European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Authorized officer Hornig, H

Form PCT/ISA/210 (second sheet) (July 1992)

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	S. KEIL AND H. KEIL: "Construction of a cassette enabling regulated gene expression in the presence of aromatic hydrocarbons" PLASMID, vol. 27, no. 3, May 1992, ACADEMIC PRESS INC., NEW YORK, US, pages 191-199, XP002047384 cited in the application see the whole document ---	1,2,5,6, 9-15
Y	J.L. RAMOS ET AL.: "Broad-host-range expression vectors containing manipulated meta-cleavage regulatory elements of the TOL plasmid" FEBS LETTERS, vol. 226, no. 2, January 1988, ELSEVIER, AMSTERDAM, NL, pages 241-246, XP002047385 cited in the application see the whole document ---	1,2,5,6, 9-15
A	HAUGAN, KAARE ET AL: "The host range of RK2 minimal replicon copy-up mutants is limited by species-specific differences in the maximum tolerable copy number" PLASMID (1995), 33(1), 27-39 CODEN: PLSMDX;ISSN: 0147-619X, 1995, XP002047386 cited in the application see the whole document ---	1-15
A	R.H. DURLAND ET AL.: "Mutations in the trfA replication gene of the broad-host-range plasmid RK2 result in elevated plasmid copy number" J. BACTERIOL., vol. 172, no. 7, July 1990, AM. SOC. MICROBIOL.,BALTIMORE,US;, pages 3859-3867, XP002047387 cited in the application see the whole document ---	1-15
A	HAUGAN K ET AL: "THE PHENOTYPES OF TEMPERATURE-SENSITIVE MINI-RK2 REPLICONS CARRYING MUTATIONS IN THE REPLICATION CONTROL GENE TRFA ARE SUPPRESSED NONSPECIFICALLY BY INTRAGENIC COP MUTATIONS." J BACTERIOL 174 (21). 1992. 7026-7032. CODEN: JOBAAY ISSN: 0021-9193, XP002047388 cited in the application see the whole document ---	1-15

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C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	VALLA, SVEIN ET AL: "Isolation and properties of temperature-sensitive mutants of the trfA gene of the broad host range plasmid RK2" PLASMID (1991), 25(2), 131-6 CODEN: PLSMDX;ISSN: 0147-619X, 1991, XP002047389 cited in the application see the whole document ---	1-15
A	C. MICHAN ET AL.: "Identification of critical amino-terminal regions of XylS" J. BIOL. CHEM., vol. 267, no. 32, 15 November 1992, AM. SOC. BIOCHEM. MOL.BIOL.,INC.,BALTIMORE,US, pages 22897-22901, XP002047390 cited in the application see the whole document ---	1-15
A	J.L. RAMOS ET AL.: "Altered effector specificities in regulators of gene expression: TOL plasmid xylS mutants and their use to engineer expansion of the range of aromatics degraded by bacteria" PROC. NATL. ACAD. SCI., vol. 83, November 1986, NATL. ACAD. SCI.,WASHINGTON,DC,US;, pages 8467-8471, XP002047391 see the whole document ---	1-15
A	N. MERMOD ET AL.: "Vector for regulated expression of cloned genes in a wide range of gram-negative bacteria" J. BACTERIOL., vol. 167, no. 2, August 1986, AM. SOC. MICROBIOL.,BALTIMORE,US;, pages 447-454, XP002047392 cited in the application see the whole document ---	1-15
A	DITTA G ET AL: "PLASMIDS RELATED TO THE BROAD HOST RANGE VECTOR, PRK290, USEFULL FOR GENE CLONING AND FOR MONITORING GENE EXPRESSION" PLASMID, vol. 13, 1 January 1985, pages 149-153, XP000106567 see the whole document ---	1-15

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C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	SCHMIDHAUSER T J ET AL: "REGIONS OF BROAD-HOST-RANGE PLASMID RK2 INVOLVED IN REPLICATION AND STABLE MAINTENANCE IN NINE SPECIES OF GRAM-NEGATIVE BACTERIA" JOURNAL OF BACTERIOLOGY, vol. 164, no. 1, 1 October 1985, pages 446-455, XP000106689 cited in the application see the whole document	1-15
A	WO 96 08572 A (RHONE POULENC RORER SA ;CAMERON BEATRICE (FR); CROUZET JOEL (FR)) 21 March 1996 see the whole document	1-15
P,X	BLATNY, JANET MARTHA ET AL: "Construction and use of a versatile set of broad-host-range cloning and expression vectors based on the RK2 replicon" APPL. ENVIRON. MICROBIOL. (1997), 63(2), 370-379 CODEN: AEMIDF;ISSN: 0099-2240, 1997, XP002047393 see the whole document	1-15
T	BLATNY J M ET AL: "Improved broad-host-range RK2 vectors useful for high and low regulated gene expression levels in Gram-negative bacteria." PLASMID 38 (1). 1997. 35-51. ISSN: 0147-619X, XP002047394 see the whole document	1-15

1

INTERNATIONAL SEARCH REPORT

nformation on patent family members

Int tional Application No
PCT/GB 97/02323

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 8909823 A	19-10-89	EP 0414713 A	06-03-91
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		DE 69104357 D	03-11-94
		DE 69104357 T	16-02-95
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		ES 2060385 T	16-11-94
		JP 6501379 T	17-02-94
		US 5670343 A	23-09-97
EP 0443063 A	28-08-91	NONE	
WO 9608572 A	21-03-96	FR 2724665 A	22-03-96
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		CA 2197804 A	21-03-96
		EP 0781341 A	02-07-97
		FI 971100 A	14-03-97
		NO 971139 A	12-03-97
		ZA 9507750 A	24-04-96